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1) Expression of nerve growth factors in pancreatic neural tissue and pancreatic cancer .

Schneider M B; Standop J; Ulrich A; Wittel U; Friess H; Andren-Sandberg A ; Pour P M

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2) Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways.

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3) Signal transduction pathways through TRK-A and TRK-B receptors in human neuroblastoma cells.

Sugimoto T; Kuroda H; Horii Y; Moritake H; Tanaka T; Hattori S
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Japanese journal of cancer research - Gann (Japan) Feb 2001 , 92 (2) p152-60, ISSN 0910-5050--Print Journal Code: 8509412

4) The trkB tyrosine protein kinase is a receptor for neurotrophin-4.

Klein R; Lamballe F; Bryant S; Barbacid M
Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000.

Neuron (UNITED STATES) May 1992 , 8 (5) p947-56, ISSN 0896-6273--Print Journal Code: 8809320

5) Neurotrophin and neurotrophin-receptor expression in primitive neuroectodermal brain tumors

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6) Relevant genomics of neurotensin receptor in cancer (ABSTRACT AVAILABLE)

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Nerve Growth Factor Stimulates Proliferation and Survival of Human Breast Cancer Cells through Two Distinct Signaling Pathways*

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We show here that the neurotrophin nerve growth factor (NGF), which has been shown to be a mitogen for breast cancer cells, also stimulates cell survival through a distinct signaling pathway. Breast cancer cell lines (MCF-7, T47-D, BT-20, and MDA-MB-231) were found to express both types of NGF receptors: p140^{trkA} and p75^{NTR}. The two other tyrosine kinase receptors for neurotrophins, TrkB and TrkC, were not expressed. The mitogenic effect of NGF on breast cancer cells required the tyrosine kinase activity of p140^{trkA} as well as the mitogen-activated protein kinase (MAPK) cascade, but was independent of p75^{NTR}. In contrast, the anti-apoptotic effect of NGF (studied using the ceramide analogue C2) required p75^{NTR} as well as the activation of the transcription factor NF- κ B, but neither p140^{trkA} nor MAPK was necessary. Other neurotrophins (BDNF, NT-3, NT-4/5) also induced cell survival, although not proliferation, emphasizing the importance of p75^{NTR} in NGF-mediated survival. Both the pharmacological NF- κ B inhibitor SN50, and cell transfection with IkBm, resulted in a diminution of NGF anti-apoptotic effect. These data show that two distinct signaling pathways are required for NGF activity and confirm the roles played by p75^{NTR} and NF- κ B in the activation of the survival pathway in breast cancer cells.

neurotrophin superfamily, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, and NT-6 (1). NGF interacts with two classes of membrane receptor: the TrkA proto-oncogene product p140^{trkA}, which possesses intrinsic tyrosine kinase activity, and a secondary receptor, p75^{NTR}, that belongs to the tumor necrosis factor (TNF) receptor family (2). The stimulation of cell survival and cell differentiation by NGF and other neurotrophins have been described primarily in neuronal cell systems (3). Although the neurotrophic effect through p140^{trkA} is known to involve the MAPK cascade, the role of p75^{NTR} is still controversial; there is evidence that it can both positively and negatively regulate neuronal cell death and differentiation, depending on the cell type examined (4). In some cases, p75^{NTR} is an inducer of apoptosis, even without NGF stimulation (5), whereas in other cases the activation of p75^{NTR} by NGF results in a protection from cell death (6). In addition to its neurotrophic function, other activities of NGF have been described. For example, NGF can modulate gene expression in monocytes (7), it is chemotactic for melanocytes (8), and its inhibition on p75^{NTR} can block the migration of Schwann cells (9). NGF also stimulates the proliferation of chromaffin cells (10), lymphocytes (11), and keratinocytes (12). We have previously shown that NGF is mitogenic for cancerous but not normal human breast cells (13), and these data, as well as others showing a role for NGF in the stimulation of prostatic cancer cells (14–17), implicate NGF in non-neuronal carcinogenesis.

Both cellular proliferation as well as tumor cell survival are crucial for malignant progression. The effect of NGF on the survival of cancer cells through the p75^{NTR} receptor has been shown for neuroblastoma (18) and schwannoma (6). In prostate cancer, p75^{NTR} has been shown to be a mediator of NGF's effects during critical phases of developmental cell death and carcinogenic progression (19). To date only the mitogenic effect of NGF for breast cancer cells has been described (13), with its roles in the control of breast cancer cell survival unknown.

In this study, we have shown that, in addition to its mitogenic effect, NGF is also an anti-apoptotic factor for breast cancer cells. These cells express mRNA for both p140^{trkA} and p75^{NTR} receptors. Our results indicate that the mitogenic effect of NGF requires p140^{trkA} and the MAPK cascade, but not the p75^{NTR} receptor, whereas the promotion of cell survival strictly requires p75^{NTR} as well as NF- κ B, but not p140^{trkA} and MAPK. Thus the mitogenic and anti-apoptotic effects of NGF on breast

Nerve growth factor (NGF)¹ is the archetypal member of the

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¹ The abbreviations used are: NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; NF- κ B, nuclear factor- κ B; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; PARP, polyADP-ribose polymerase; TNF, tumor necrosis factor; TBP, TATA box binding protein; RT-PCR, reverse transcriptase-polymerase chain reaction; FCS, fetal calf serum; DTT, dithiothreitol; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; GFP, green fluorescence protein; IkBm, dominant-negative IkBa mutant; bp, base pair(s); PD98059, Park Davis 98059.

cancer cells are mediated through two different signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from BioWhittaker (France) except insulin, which was obtained from Organon (France). Recombinant human nerve growth factor, brain derived growth factor (BDNF), and neurotrophins 3 (NT-3) and 4 (NT-4) were from R & D Systems (UK). K-252a (inhibitor of trk-tyrosine kinase activity) and PD98059 (inhibitor of MAPK cascade) were from Calbiochem (France). The mouse monoclonal anti-NGF receptor (p75^{NTR}) antibody was from Euromedex (France) and was previously described for its ability to block the interaction between p75^{NTR} and NGF (20). The anti-lamin B (C-20), goat polyclonal IgG, and the polyclonal anti-p140^{trkA} (trk763) were from Santa Cruz Biotechnology. C2 ceramide analogue (*N*-acetyl-D-sphingosine), Hoechst 33258, and electrophoresis reagents were from Sigma Chemical Co. (France). The SN50 NF- κ B inhibitor peptide, the rabbit polyclonal anti-NF- κ B p65 antibody, was obtained from TEBU (France). Anti-PARP antibody was from Oncogene Research Products (UK). Primers and probes for TrkA and p75^{NTR}, probe for TATA box binding protein (TBP) were from Eurogentec (Belgium). RT-PCR reagents were from Applied Biosystems (France). Lipofectin reagent and Opti-MEM were provided by Life Technologies, Inc. (France). The green fluorescence protein plasmid (EGFP-C1) was purchased from CLONTECH, and the dominant-negative I κ B α mutant (I κ Bm) expression vectors (in pCDNA3) containing a Ser to Ala substitution at residues 32 and 36 were obtained from Dr. Jean Feuillard (UPRES EA 1625, Bobigny, France). p65 (rel-A) and c-rel cDNA were cloned at *Eco*RI site in PSVK3 expression plasmid. All vectors were obtained from Dr. Pascale Crépiaux (McGill University, Montreal). The SY5Y subclone of SK-N-SH neuroblastoma cell line was a kind gift of Dr. Luc Buée (INSERM, U422, Lille, France). NT-2 (Ntera/D1) human neural precursor cells (Stratagene) are derived from a clone of the NT-2 teratocarcinoma.

Cell Culture—Breast cancer cell lines (MCF-7, T47-D, BT-20, and MDA-MB-231) were obtained from the American Type Culture Collection and routinely grown as monolayer cultures. Cells were maintained in minimal essential medium (Earle's salts) supplemented with 20 mM Hepes, 2 g/liter sodium bicarbonate, 2 mM L-glutamine, 10% fetal calf serum (FCS), 100 units/ml penicillin-streptomycin, 50 μ g/ml gentamicin, 1% of non-essential amino acids, and 5 μ g/ml insulin.

Detection of Neurotrophin Receptors mRNA Expression—The reverse transcription reaction mixture contained 2 g of purified total RNA (extracted from breast cancer cell lines, NT-2 cells, or SY5Y cells), 1 \times reverse transcription reaction buffer, 10 mM DTT, 400 mM dNTP each, 2.5 M oligo(dT) 18 primer, 40 units of RNasin, and 200 units of Moloney murine leukemia virus reverse transcriptase were added to 25 μ l of total reaction volume. All the reaction mixtures were incubated at 37 °C for 1 h and then inactivated at 95 °C for 5 min. Polymerase chain reaction was performed on cDNAs after RT or corresponding total RNA samples without the RT step for negative controls. The primers used for *trkA* and p75 RT-PCR detection in breast cancer cell lines were as follows: *trkA* sense primer, 5' (291)-CATCGTGAAGAGTGTCTCCG-3' (311) and antisense primer, 5' (392)-GAGAGAGACTCCAGAGCGTTGAA-3' (370) or p75 sense primer, 5' (442)-CCTACGGCTACTACCAGATGAG-3' (462) and antisense primer, 5' (588)-TGGCCTCGTCGGAATACG-3' (571). The primers used for RT-PCR comparative detection of trks in MCF-7 cells were as follows: *trkA* sense primer, 5' (118)-AGGCGGTCTGGTGACTTCGTG-3' (139) and antisense primer, 5' (1162)-GGCAGCCAGCAGGGTGTAGTTC-3' (1141) or *trkB* sense primer, 5' (134)-CGAGGTTGGAACCTAACAGCATTG-3' (157) and antisense primer, 5' (1182)-GTCAGTTGGCGTGGTCCAGTCTTC-3' (1159) or *trkC* sense primer, 5' (219)-CACGGACATCTCAAGGAAGAGCA-3' (241) and antisense primer, 5' (1078)-CTGAGAACTTCAACCTCCTGGTAG-3' (1056). Each pair of primers was used in RT-PCR reaction to amplify trks or p75. To PCR tubes were added 5 μ l of PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 10 μ l of 15 mM MgCl₂, 1 μ l of 10 mM dNTP mix, 1 μ l of cDNA or total mRNA (for negative control), 1 μ l of 50 mM respective primers, 1 μ l of 2.5 units/ μ l *Taq* DNA polymerase, and water to a total volume of 50 μ l. The PCR conditions were as follows: after 95 °C for 3 min for denaturing cDNA. 30 cycles were run at 94 °C for 1 min, 57 °C for 2 min, and 72 °C for 3 min. The PCR tubes were incubated for a further 10 min at 72 °C for the extension of cDNA fragments after the final cycle, and the PCR products were electrophoresed in an agarose gel.

Cell Growth Assay—Experiments were performed as previously described (13). 35-mm diameter dishes were inoculated with 2 \times 10⁴ cells/dish in 2 ml of medium containing 10% FCS. After 24 h, cells were

washed twice with serum-free medium. Next day, the medium was replaced with 2 ml of serum free medium containing 100 ng/ml NGF or various concentrations of other neurotrophins (BDNF, NT-3, NT-4/5). To study the effect of pharmacological inhibitors or blocking antibodies, various concentrations were added simultaneously with NGF (100 ng/ml). After 2 days of NGF exposure, cells were harvested by trypsinization and counted using an hemocytometer.

Determination of the Percentage of Apoptotic Cell Nuclei—Apoptosis of breast cancer cells was induced by the ceramide analogue C2, which has been described as a pro-apoptotic agent for human breast cancer cells (21, 22). Apoptosis was obtained by treatment with 2 μ M C2 for 24 h. To evaluate the anti-apoptotic activity of NGF, various concentrations of this factor were tested; we found that the maximal effect was obtained for 100 ng/ml. Consequently, this concentration was used in all experiments with pharmacological inhibitors or blocking antibody. For determination of apoptotic cell percentage, cells were fixed with cold methanol (−20 °C) for 10 min and washed twice with phosphate-buffered saline (PBS) before staining with 1 μ g/ml Hoechst 33258 for 10 min at room temperature in the dark. Cells were then washed with PBS and mounted with coverslips using Glycergel (Dako). The apoptotic cells exhibiting condensed and fragmented nuclei were counted under an Olympus-BH2 fluorescence microscope in randomly selected fields. A minimum of 500–1000 cells was examined for each condition, and results were expressed as a ratio of the total number of cells counted.

Statistical Analysis and Software—The statistical analysis of the data gathered from cell and apoptotic nuclei counting was performed using SPSS version 9.0.1 (SPSS inc., Chicago, IL). Analyses of variance were followed by the Tukey's test to determine the significance.

NGF Receptors and PARP Immunoblotting—Subconfluent cell cultures were harvested by scraping in serum-free medium. After centrifugation (1000 \times g, 5 min), the pellet was treated with lysis buffer (0.3% SDS, 200 mM dithiothreitol) and boiled 5 min. In the case of PARP, the pellet was lysed with urea-rich buffer (62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS), sonicated and incubated at 65 °C for 15 min. The lysates were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane (Immobilon-P, Millipore) by electroblotting (100 V, 75 min), and probed with anti-*trkA*, anti-p75^{NTR} or anti-PARP antibodies at 4 °C overnight. The membranes were then incubated at room temperature for 3 h with biotin-conjugated anti-rabbit (*trkA*) or anti-mouse (p75^{NTR} and PARP) immunoglobulin G. After 1 h of incubation with extravidin, the reaction was revealed using the chemiluminescence kit ECL (Amersham Pharmacia Biotech) with Kodak X-Omat AR film.

Detection of p140^{trkA} and MAPK Activation—Proteins were extracted in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% SDS, 1% Nonidet P-40, 100 μ M sodium orthovanadate) prior to immunoprecipitation. Preclearing was done with protein A-agarose (10 μ l/250 μ l, 60 min, 4 °C). After centrifugation (10,000 \times g, 2 min), the supernatant was incubated with monoclonal anti-MAPK (anti-ERK2) antibody (10 μ l/250 μ l, 60 min, 4 °C). Protein A-agarose (10 μ l) was added for 60 min (4 °C) and then pelleted by centrifugation (10,000 \times g, 2 min). The pellet was then rinsed three times with lysis buffer and boiled for 5 min in Laemmli buffer. After SDS-PAGE and electroblotting, nitrocellulose membranes were blocked with 3% bovine serum albumin. Membranes were then incubated with PY20 anti-phosphotyrosine antibody overnight at 4 °C, rinsed, and incubated with a horseradish peroxidase-conjugated anti-mouse IgG for 3 h at room temperature. Membranes were rinsed overnight at 4 °C before visualization with ECL.

Cell Fractionation and NF- κ B Detection—Cell nuclear extracts were prepared as described by Herrmann *et al.* (23). Cells were trypsinized and then pelleted in minimal essential medium containing 10% FCS. After washing with ice-cold PBS, cells were repelleted and resuspended in 400 μ l of ice-cold hypotonic buffer (10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 10 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, 3 mM phenylmethylsulfonyl fluoride, and 3 mM DTT). After 10 min on ice, 25 μ l of 10% Nonidet P-40 was added and crude nuclei were collected by centrifugation for 5 min. The nuclear pellet was resuspended in high salt buffer (50 mM Hepes, pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, 3 mM DTT, and 3 mM phenylmethylsulfonyl fluoride). After 30 min on ice with frequent agitation, the insoluble nuclear material was pelleted in a microfuge for 10 min. Crude nuclear protein was collected from the supernatant and snap-frozen in a dry ice/ethanol bath. After thawing and boiling for 5 min in Laemmli buffer, the nuclear extracts were subjected to SDS-PAGE and probed with an anti-NF- κ B p65 antibody. A control was established with anti-lamin B antibody.

Transfection of I κ B, c-rel, and rel-A—Cotransfection experiments were carried out using Lipofectin reagent, as described by the manu-

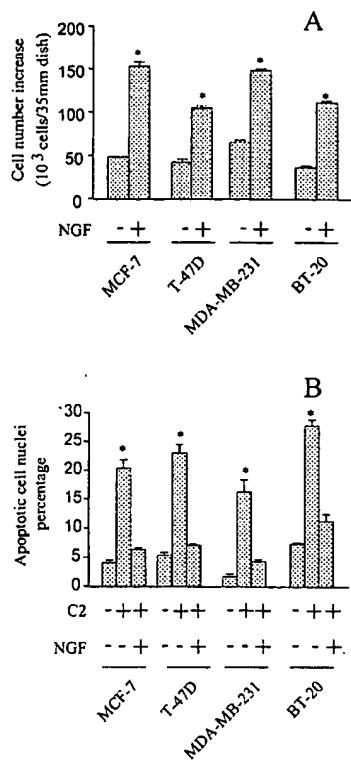


FIG. 1. Effect of NGF on the growth and survival of breast cancer cells. *A*, breast cancer cells were serum-deprived in minimum essential medium, and after 24 h the NGF (100 ng/ml) was added. After 48 h, cells were harvested and counted. *B*, cells were serum-deprived in minimum essential medium and treated with 2 μ M C2 with or without 100 ng/ml NGF. After 24 h, cells were fixed and the proportion of apoptotic nuclei were determined after Hoechst staining under an Olympus-BH2 fluorescence microscope. For measurement of both cell number and apoptosis, results are expressed as the means \pm S.D. of five separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$).

facturer. Briefly, MCF-7 cells were incubated for 5 h in 1 ml of Opti-MEM transfection medium containing 8 μ l of Lipofectin reagent, 0.8 μ g of green fluorescence protein (GFP)-carrying vector and 0.2 μ g of empty vector PCDNA3 or 0.2 μ g of *IkBm*. In the case of c-rel or rel-A, cells were cotransfected with 0.8 μ g of GFP-carrying GFP and 0.6 μ g of PSVK₃ (empty plasmid), c-rel, or rel-A. Cells were then grown for 24 h with 10% FCS minimal essential medium and rinsed for 2 h in serum-free medium before incubation in serum-free medium in the presence or absence of 100 ng/ml NGF and/or 2 μ M C2 for another 24 h. Cells were then fixed with paraformaldehyde 4% (4 $^{\circ}$ C) for 30 min, and the percentage of apoptotic cell nuclei in GFP-stained cells was determined as described above.

RESULTS

NGF Mitogenic and Anti-apoptotic Activity for Breast Cancer Cells—The effects of 100 ng/ml NGF on cell proliferation and C2-induced apoptosis were evaluated by cell counting and Hoechst staining, respectively. The results show that NGF induces an increase in cell number for all breast cancer cell lines tested (Fig. 1A). We have previously demonstrated that NGF has a direct mitogenic effect on breast cancer cells by recruiting cells in G₀ phase and by shortening the G₁ length (Descamps *et al.*, 1998). In addition, NGF rescued breast cancer cells undergoing C2-induced apoptosis; the maximum survival was observed at 200 ng/ml (Fig. 1B). The morphology of cells undergoing this NGF-induced anti-apoptotic rescue was quite distinct (Fig. 2A). The induction of apoptosis by C2 was found to involve cleavage of poly(A)DP-ribose polymerase (PARP); this cleavage was reversed by NGF (Fig. 2B).

TrkA and p75^{NTR} Expression—RT-PCR was used to show the expression of mRNA for both high and low affinity NGF recep-

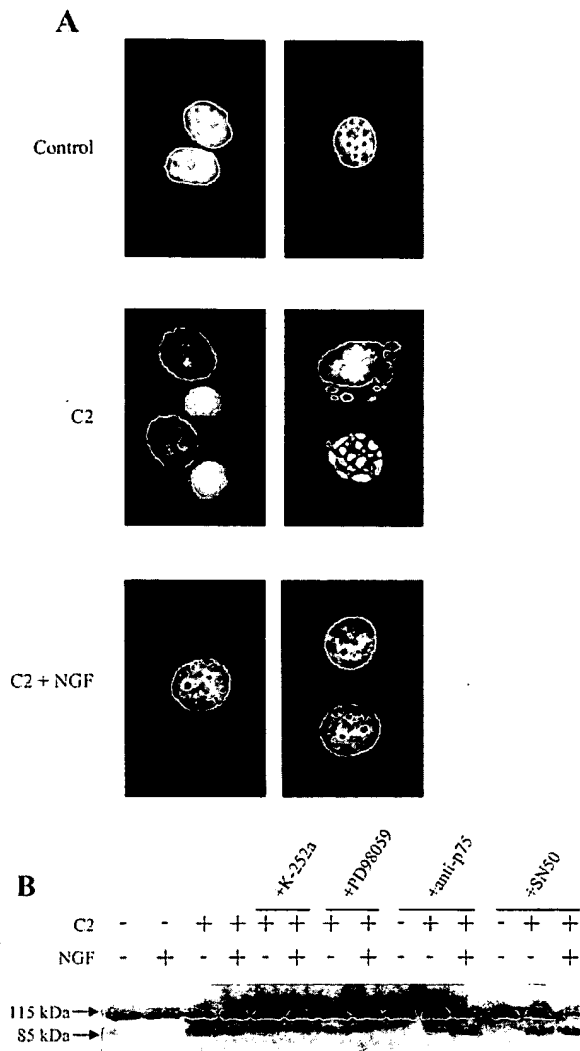


FIG. 2. Anti-apoptotic effect of NGF. *A*, Hoechst staining of apoptotic cell nuclei in control, C2 and C2+NGF-treated MCF-7 cells. Cells were serum-deprived in minimum essential medium and treated with C2. NGF was added at 100 ng/ml. After 24 h, cells were fixed and apoptotic nuclei were observed after Hoechst staining. *B*, immunoblot detection of PARP cleavage. C2-induced PARP cleavage was reversed by p75^{NTR} activation mediated by NGF. MCF-7 cells were serum-deprived in minimum essential medium for 24 h and were then treated with 100 ng/ml NGF in the presence or absence of 2 μ M C2, 10 nM K-252a, 10 μ M PD98059, or 10 μ g/ml anti-p75^{NTR}-blocking antibody (Euromedex) for another 24-h period. Proteins were detected after SDS-PAGE of cell preparations from MCF-7 breast cancer cells, electroblotting onto nitrocellulose, and immunodetection with anti-PARP antibodies.

tors in MCF-7, T47-D, BT-20, and MDA-MB-231 cells (Fig. 3A); the 102-bp band for the TrkA transcript and a 147-bp band for the p75^{NTR} transcript were readily detectable on 1% agarose gels. Moreover, Western blotting demonstrated that both p140^{TrkA} and p75^{NTR} were present in all the breast cancer cell lines (Fig. 3B). Real-time quantitative RT-PCR indicated that there was no significant change in the levels of TrkA and p75^{NTR} mRNAs in the presence of FCS, NGF, or C2 (data not shown) and that the levels of mRNA for TrkA and p75^{NTR} in breast cancer cells was between 5 and 10 times lower than the level observed in SY5Y neuroblastoma cells (data not shown). This indicates that NGF receptor expression in breast cancer cells is relatively limited. It should be noticed that, although mRNA levels of NGF receptors differ between breast cancer cells and SY5Y, the protein levels apparently do not. However,

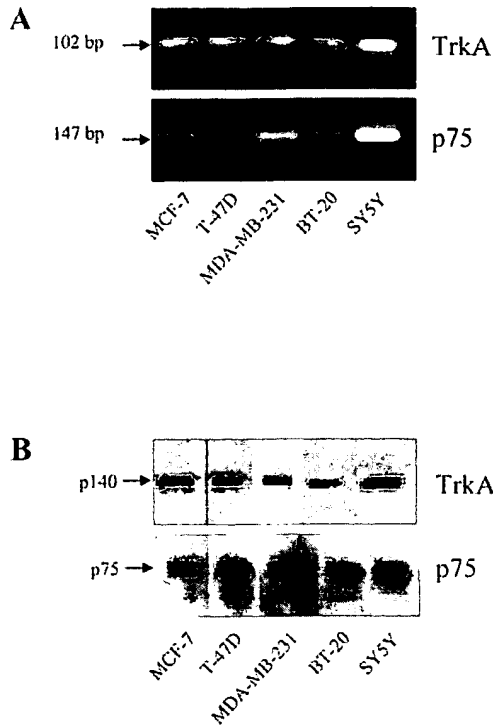


FIG. 3. TrkA and p75^{NTR} expression in breast cancer cells. A, agarose gel electrophoresis of RT-PCR products evidenced a 102-bp band and a 147-bp band, which are characteristic of TrkA and p75^{NTR}, respectively. Both NGF receptors were found in all cell types tested. B, p140^{TrkA} and p75^{NTR} were immunodetected after SDS-PAGE of breast cancer cell lines. The neuroblastoma cells SY5Y were used as positive control for the expression of NGF receptors.

it has been shown before that the level of a given cellular protein cannot be simply deduced from mRNA transcript level (24). One could hypothesize that the stability of mRNA and/or protein for NGF receptors, differs between breast cancer cells and neuroblastoma cells, leading to the observed disproportionality between mRNA and protein levels.

Involvement of p140^{TrkA} and p75^{NTR} in Mitogenic and Survival Activities of NGF—We used a combination of specific antibodies and pharmacological inhibitors to study the putative functions of p140^{TrkA} and p75^{NTR} in the stimulation of proliferation and cell survival induced by NGF. The Trk tyrosine kinase inhibitor K-252a, and the MEK inhibitor PD98059, both strongly inhibited the growth-stimulatory effect of NGF on MCF-7 cells, but had no effect on its anti-apoptotic effects (Fig. 4). Conversely, neither the anti-p75^{NTR} blocking antibody nor the NF- κ B inhibitor SN50 affected NGF-stimulated proliferation, although both strongly reduced the anti-apoptotic effects (Fig. 4). The tyrosine kinase activity of p140^{TrkA} was inhibited by K-252a but not by the anti-p75^{NTR} or PD98059 (Fig. 5). On the other hand, the activity of the MAPKs was inhibited by K-252a and PD98059 but not by the anti-p75^{NTR} (Fig. 5). It should be noted that the SN50 peptidic inhibitor of NF- κ B, similarly to the anti-p75^{NTR}, inhibited the anti-apoptotic effect of NGF but neither its proliferative effect nor its activation of p140^{TrkA} and MAPKs. The effect of other neurotrophins on MCF-7 cell growth and survival was also evaluated (Fig. 6A). In contrast to NGF, no proliferative effect was provided by BDNF, NT-3, or NT-4/5. However, all neurotrophins tested exhibited a rescue effect on C2-treated cells that was not altered in the presence of the trk inhibitor K-252a (Fig. 6B). These data suggest that trk receptors are not involved in NGF survival activity. Moreover, the participation of trkB and trkC

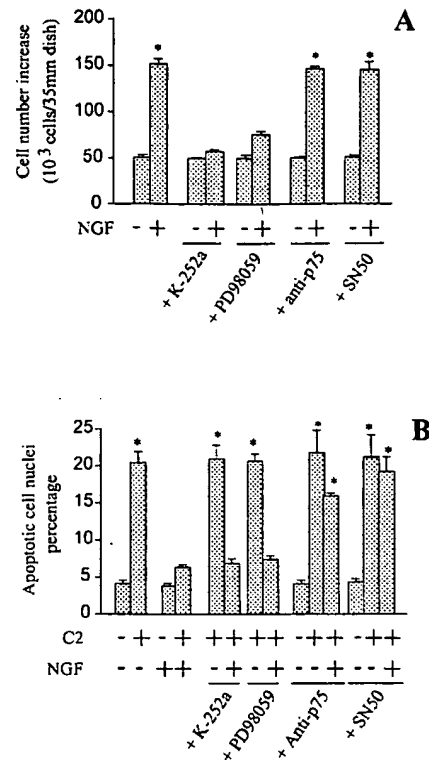


FIG. 4. Pharmacological modulation of the proliferative and anti-apoptotic effect of NGF. MCF-7 cells were starved in minimum essential medium, and after 24 h, 100 ng/ml NGF was added with or without inhibitors or antibody. A, after 48 h, cells were harvested and counted. B, after 24 h, cells were fixed and the proportion of apoptotic nuclei determined after Hoechst staining. The following concentrations were used: 2 μ M C2, 10 nM K-252a, 10 μ M PD98059, 10 μ g/ml anti-p75^{NTR} blocking antibody (Euromedex), 10 μ M SN50. For A and B, results are expressed as the means \pm S.D. of five separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$).

in these events can be ruled out, because they are not expressed in these breast cancer cells (Fig. 6C).

NF- κ B Involvement in the Anti-apoptotic Effect of NGF—The inhibitory effect of SN50 on the NGF anti-apoptotic activity indicated the potential involvement of NF- κ B in the signaling leading to the protective activity of this growth factor. To further investigate this phenomenon, we studied the effect of NGF on the nuclear translocation of NF- κ B, as well as the consequence of transfection by I κ Bm (an inhibitor of NF- κ B) or by c-rel and rel-A (constitutively active subunits of NF- κ B) on the NGF-mediated anti-apoptotic activity in MCF-7 cells. Western blotting revealed no change in the nuclear levels of NF- κ B (p65) during apoptosis induced by C2 (Fig. 7). In contrast, the addition of NGF on C2-treated cells induced a translocation of NF- κ B from cytoplasm to nucleus. Computerized quantification revealed a doubling p65 band intensity normalized to the total intensity of the lane (data not shown). Moreover, this NF- κ B nuclear translocation was inhibited by the presence of p75^{NTR}-blocking antibody or SN50, but was not affected by K-252a and PD98059. Interestingly, in the absence of C2-induced apoptosis NGF was not able to induce the nuclear translocation of NF- κ B, confirming previous observations that p75^{NTR}-mediated NF- κ B activation requires cell stress (25). Transfection of MCF-7 cells with I κ Bm, an inhibitor of NF- κ B, reversed the anti-apoptotic effect of NGF (Fig. 8A). As a control, we transfected MCF-7 cells with an empty vector; no effect was observed. In addition, transfection with activators of the NF- κ B pathway, c-rel or rel-A (Fig. 8B), resulted in an inhibition of C2-induced apoptosis of MCF-7 cells, even in absence of NGF,

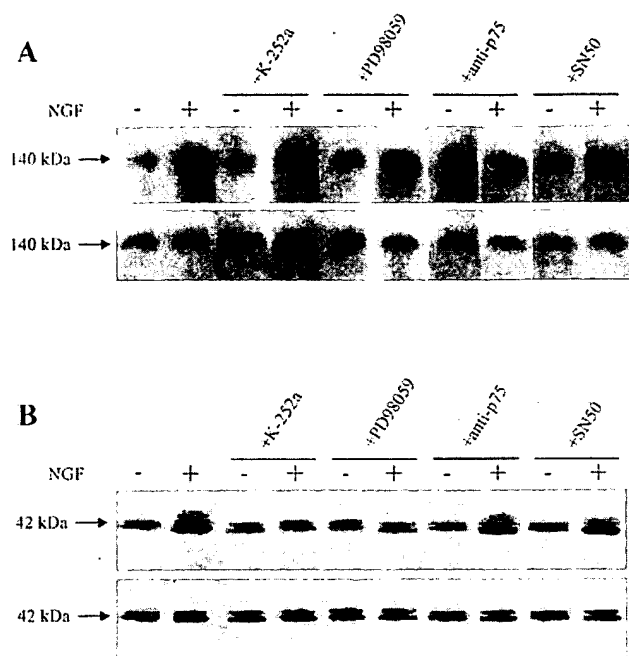


FIG. 5. **p140^{trkA} and MAPK activation.** MCF-7 cells were treated with 100 ng/ml NGF in the presence or absence of 10 nM K-252a, 10 μ g/ml anti-p75^{NTR}-blocking antibody, or 10 μ M PD98059. p140^{trkA} (A) and MAPK activation (B) were determined after immunoprecipitation using polyclonal anti-TrkA and monoclonal anti-ERK2 antibodies, respectively. After SDS-PAGE and electroblotting, nitrocellulose membranes were counterprobed with the PY20 anti-phosphotyrosine antibody. For detection of TrkA (A) and MAPK (B) activation, the lower panel shows reprobing of the blots with the immunoprecipitating antibody.

confirming the involvement of NF- κ B family members in human breast cancer cell survival.

DISCUSSION

This study shows that, in addition to its mitogenic activity, NGF is anti-apoptotic for breast cancer cells, and that these two biological effects are differentially mediated by the p140^{trkA} and p75^{NTR} receptors, respectively. The growth of breast cancer results from a balance between cell proliferation and apoptosis, both of which can be modulated by various regulatory peptides. For example, epidermal growth factor, fibroblast growth factors, and insulin-like growth factor-1 can all stimulate the proliferation and survival of breast cancer cells (26). On the other hand, agents such as transforming growth factor- β or tumor necrosis factor- α can inhibit growth and induce apoptosis in these cells (27). Recently we have shown that NGF, which was primarily described for its neurotrophic properties, is a strong mitogen for cancerous but not for normal human breast epithelial cells, suggesting a crucial function for this factor in the initiation and progression of human breast tumors (13). In the present study, we have shown that the breast cancer cells express transcripts for both TrkA and p75^{NTR} receptors. In contrast, no expression of TrkB and TrkC was found in any of the breast cancer cells tested, in accordance with the fact that BDNF, NT-3, or NT-4/5 have no mitogenic effect for these cells. The presence of NGF receptors has been detected previously in breast cancer cells (28), and low levels of NGF receptor expression have recently been reported in other breast cancer cell lines (29), leading to the hypothesis of a recruitment and cooperation between p140^{trkA} and p185^{Her-2} for the induction of mitogenesis by NGF. Our results indicate a stimulation of p140^{trkA} tyrosine kinase activity and of the MAPK cascade by NGF, and the use of the pharmacological inhibitors K-252a and PD98059 demonstrate the requirement

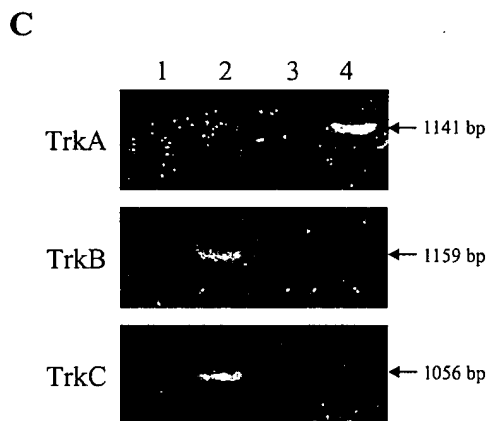
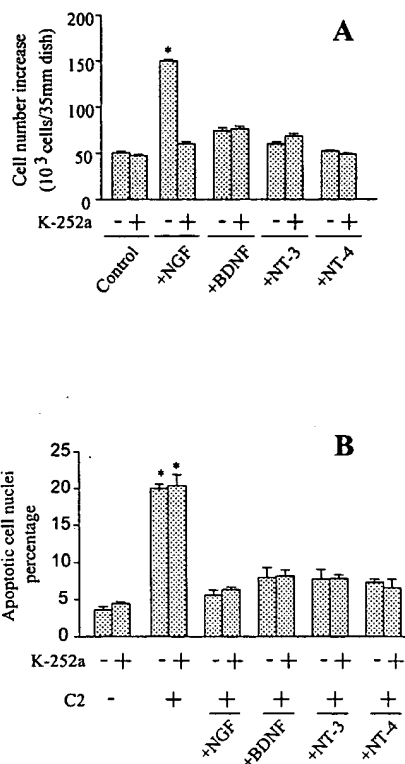


FIG. 6. **Effect of different neurotrophins on MCF-7 cells growth and survival.** MCF-7 cells were serum-deprived in minimum essential medium, and after 24 h the neurotrophins (100 ng/ml NGF, 50 ng/ml BDNF, 50 ng/ml NT-3, 100 ng/ml NT-4/5) were added. A, after 48 h, cells were harvested and counted. In contrast with NGF, neither BDNF, NT-3, nor NT-4/5 displayed significant bioactivity (for concentrations up to 400 ng/ml). B, MCF-7 cells were serum-deprived in minimum essential medium and treated with 2 μ M C2, with or without neurotrophins (100 ng/ml NGF, 50 ng/ml BDNF, 50 ng/ml NT-3, 100 ng/ml NT-4/5). After 24 h, cells were fixed and apoptotic nuclei percentage was determined after Hoechst staining under an Olympus-BH2 fluorescence microscope. For measurement of both cell number and apoptosis, results are expressed as the means \pm S.D. of five separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$). C, TrkB and TrkC mRNA expression in MCF-7 cells. Agarose gel electrophoresis of RT-PCR products reveals TrkA expression, but no TrkB or TrkC expression in MCF-7 breast cancer cells. Human NT2 cells were used as positive control for the expression of TrkB and TrkC. Lane 1, NT2-negative control without RT step; lane 2, NT2-positive control; lane 3, MCF-7 cells-negative control without RT step; lane 4, MCF-7 cells.

for these signals in NGF-induced MCF-7 cell proliferation. The induction of MAPK activity required p140^{trkA} activation, but p75^{NTR} did not appear to be involved, because p75^{NTR}-blocking antibodies did not have any effect on NGF-induced MAPK

FIG. 7. Activation of NF- κ B during NGF anti-apoptotic effect. MCF-7 cells were treated with 100 ng/ml NGF in the presence or absence of 10 nM K-252a, 10 μ M PD98059, or 10 μ g/ml anti-p75^{NTR} blocking antibody (Euromedex). Proteins were detected after SDS-PAGE of nuclear extract preparations and immunoblotting with rabbit anti-NF- κ B p65. The lower panel shows immunoblotting with anti-lamin B.

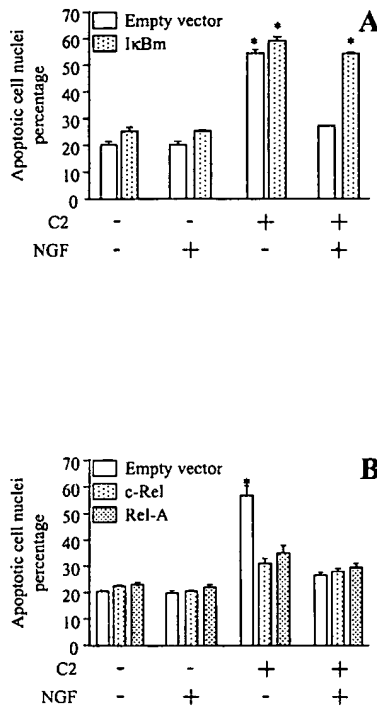
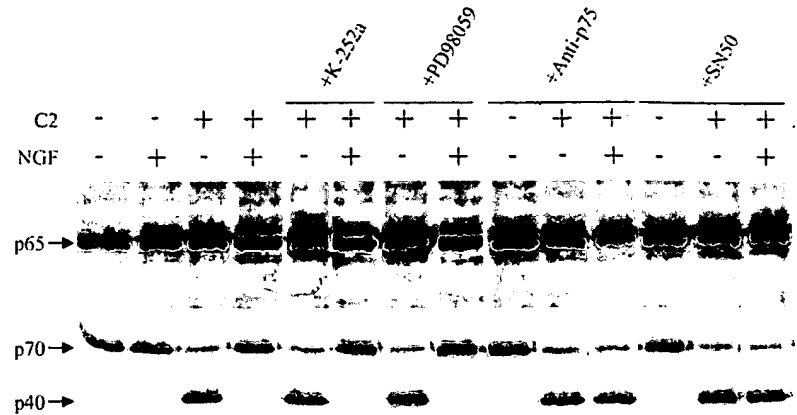


FIG. 8. Modulation of NGF anti-apoptotic effect by I κ Bm, c-rel, and rel-A transfection. MCF-7 cells were co-transfected with EGFPc1 and either I κ Bm (A) or c-rel or rel-A (B) using the Lipofectin reagent. Controls were performed with both PCDNA3 and EGFPc1 (for I κ Bm experiments) and both PSVK₂ and EGFPc1 (for c-rel and rel-A experiments). After 24 h, cells were serum-deprived in minimum essential medium and either C2 or neurotrophins added for another 24-h period. Cells were then fixed and the percentage of apoptotic nuclei in transfected cells (with the expression of GFP as a transfection control) determined after Hoechst staining. Results are expressed as the means \pm S.D. of six separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$).

activation and cell proliferation. In contrast, p75^{NTR}-blocking antibodies exhibited an inhibition of NGF-induced survival, attesting to the functionality of these blocking antibodies. Thus, the mitogenic activity of NGF requires the p140^{trkA} and MAPK cascade independently of the p75^{NTR} receptor. This signaling pathway for the NGF-proliferative effect appears to be similar to that which is described for the neurotrophic activity of this factor. For example, in PC-12 pheochromocytoma cells, disruption of p75^{NTR} does not result in an inhibition of the NGF differentiative activity, which is mediated by the p140^{trkA}/MAPK pathway (30). Interestingly, it has also been shown in PC-12 cells that NGF induces survival and differentiation through two distinct signaling pathways, because the activation of the MAPK cascade is required for the differentia-



tive but not the protective activity of NGF (31). These data emphasize the similarities between the mitogenic and neurotrophic signaling pathways of NGF.

The function of NGF as a survival factor has been extensively described for neurons in both *in vitro* and *in vivo* (32). However, the intracellular signaling involved in the anti-apoptotic activity of NGF in neurons remains controversial. The p140^{trkA}/MAPK cascade is generally described as protective for neuronal cell death, although there has been a recent report of a novel apoptotic pathway mediated by p140^{trkA}/MAPK in medulloblastoma cells (33). Unlike the p140^{trkA} receptors, the definition of the precise physiological role of p75^{NTR} has proven difficult (4). The p75^{NTR} receptor belongs to the TNF-receptor family, including among others, types I and II of the TNF receptor, the Fas antigen, and CD40 (34). The common cellular responses to activation of this family of receptors are the activation of gene transcription via nuclear factor- κ B (NF- κ B) and the regulation of cell survival/apoptosis. In some cases apoptosis was shown to develop following NGF binding to p75^{NTR}, although in other cases it appeared to occur in the absence of ligand (spontaneous apoptosis) and was reversed by NGF (35). The C2 reagent used here is known to induce apoptosis in breast cancer cells such as MCF-7 (21, 22). Morphological analysis after Hoechst staining and the inhibition of PARP cleavage demonstrated that NGF rescues breast cancer cells from C2-induced cell death. Interestingly, K-252a and PD98059 did not affect the anti-apoptotic activity of NGF, indicating that p140^{trkA} tyrosine kinase and MAPK activities are not necessary for the protective effect. Previous reports have noted that NGF is able to elicit its biological effects through p75^{NTR} receptors and independently of p140^{trkA} in neurons (36, 37) and Schwann cells (38). In our experiments, a specific role for p75^{NTR} in the cell survival effect was first suggested by the fact that other neurotrophins (interacting with p75^{NTR} and not with p140^{trkA}) are also able to protect cells from death while having no impact on cell proliferation. The crucial role of p75^{NTR} was further demonstrated by the use of p75^{NTR}-blocking antibodies, which completely reversed the protective effect of NGF from C2-induced apoptosis. Moreover, BDNF, NT-3, and NT-4/5, all of which can bind p75^{NTR}, can also stimulate breast cancer cell survival. Because TrkB and TrkC are not expressed in breast cancer cells, these data emphasize the role played by p75^{NTR} in the anti-apoptotic effect of NGF. Activation of p75^{NTR} specifically induces NF- κ B independent of p140^{trkA} in several cell types, including Schwann cells (38). To explore the involvement of NF- κ B in the NGF survival effect, we first tested SN50, which inhibits the nuclear translocation of this transcription factor (39). We found that it blocked the anti-apoptotic effect of NGF without affecting the p140^{trkA}/MAPK cascade or cellular proliferation. The involvement of

NF- κ B was further demonstrated by transfection with a mutated form of I κ B α , which blocked NF- κ B translocation to the nucleus. MCF-7 cells transfected by mutated I κ B α were not rescued from C2-induced apoptosis by NGF, confirming the involvement of NF- κ B in the anti-apoptotic activity mediated by p75^{NTR}. Similar observations have been made in PC12 cells in which the blocking of p75^{NTR}-mediated activation of NF- κ B resulted in an enhancement of apoptosis (40). In addition, transfections by c-rel or rel-A, which are constitutively activated forms of NF- κ B, had a protective effect on MCF-7 cells treated by C2 in absence of NGF stimulation. c-rel and rel-A belong to the NF- κ B family of transcription factors. The protection from apoptosis observed after transfection with this factor emphasizes the role played by NF- κ B molecules in the control of breast cancer cell survival.

In conclusion, our results demonstrate that NGF is an anti-apoptotic factor for human breast cancer cells and that the signaling pathway leading to this survival activity is distinct from the signaling pathway, which leads to mitogenic stimulation. Although p140^{trkA} and the MAPKs mediate the mitogenic activity of NGF, its anti-apoptotic activity required p75^{NTR} and NF- κ B alone. NGF is present in the mammary gland (41, 42) as well as its transcripts,² and our present finding therefore emphasizes that NGF is a crucial regulator of mammary tumor growth. The inhibition of breast cancer progression through the targeting p140^{trkA} and p75^{NTR} should be considered as a potential perspective for the treatment of this pathology.

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7) Expression and function of TRK-B and BDNF in human neuroblastomas.

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Department of Pathology, MCP-Hahnemann University, Philadelphia, PA, USA.

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9) Differential cellular expression of neurotrophins in cortical tubers of the tuberous sclerosis complex.

Kyin R; Hua Y; Baybis M; Scheithauer B; Kolson D; Uhlmann E; Gutmann D;
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10) Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina.

Ghazi-Nouri S M S; Assi A; Limb G A; Scott R A H; von Bussmann K;
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11) Expression of NGF family and their receptors in gastric carcinoma: a cDNA microarray study.

Du Jian-Jun; Dou Ke-Feng; Peng Shu-You; Qian Bing-Zhi; Xiao Hua-Sheng;
Liu Feng; Wang Wei-Zhong; Guan Wen-Xian; Gao Zhi-Qing; Liu Ying-Bin; Han
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12) ssociation of neurotrophin receptor expression and differentiation in human neuroblastoma.

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13) Expression of brain-derived neurotrophic factor and tyrosine kinase B receptor proteins in glioneuronal tumors from patients with intractable epilepsy: colocalization with N-methyl-D-aspartic acid receptor.

Aronica E; Leenstra S; Jansen G H; van Veelen C W; Yankaya B; Troost D

Differential Cellular Expression of Neurotrophins in Cortical Tubers of the Tuberous Sclerosis Complex

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Neurotrophins and their receptors modulate cerebral cortical development. Tubers in the tuberous sclerosis complex (TSC) are characterized histologically by disorganized cortical cytoarchitecture and thus, we hypothesized that expression of neurotrophin mRNAs and proteins might be altered in tubers. Using *in situ* transcription and mRNA amplification to probe cDNA arrays, we found that neurotrophin-3 (NT3) and *trkB* mRNA expression were reduced whereas neurotrophin-4 (NT4) and *trkC* mRNA expression were increased in whole tuber sections. Alterations in mRNA abundance were defined in single microdissected dysplastic neurons (DNs) and giant cells (GCs). NT3 mRNA expression was reduced in GCs and *trkB* mRNA expression was reduced in DNs. NT4 mRNA expression was increased in DNs and *trkC* mRNA expression was increased in both DNs and GCs. In three patients, *TSC2* locus mutations were confirmed and the mean tuberin mRNA expression levels was reduced across all nine cases. Consistent with these observations, NT3 mRNA expression was reduced but *trkC* mRNA expression was increased *in vitro* in human NTera2 neurons (NT2N) transfected with a tuberin antisense construct that reduced tuberin expression. Western analysis of tuber homogenates and computer-assisted densitometry of immunolabeled sections confirmed the neurotrophin mRNA expression data in whole sections and single neurotrophin immunoreactive cells. We conclude that alterations in NT4/*trkB* and NT3/*trkC* expression may contribute to tuber formation during brain development as downstream effects of the hamartin and tuberin pathway in TSC. (*Am J Pathol* 2001; 159:1541-1554)

Tubers in the tuberous sclerosis complex (TSC) are developmental abnormalities of cerebral cortical cytoarchitecture that are associated clinically with epilepsy.¹⁻³ Electrocorticography has shown that tubers are epileptogenic and seizures in TSC patients are often medically intractable despite anticonvulsant polytherapy.⁴⁻⁶ TSC is an autosomal-dominant disorder resulting from mutations in one of two genes, *TSC1* or *TSC2*,^{7,8} although the mechanism by which mutations in either TSC gene leads to tuber formation is unknown. Disorganized cortical lamination and aberrant cellular morphology are important histological features of tubers. Large dysplastic neurons (DNs) and giant cells (GCs) are prominent cell types in tubers.⁹ DNs and GCs share select morphological features including cytomegaly, the extension of aberrant processes often of unclear identity, ie, axons versus dendrites, and the expression of neural protein markers such as neurofilament and α -internexin.^{1,9,10}

The *TSC2* knockout mouse¹¹ and the Eker rat strain¹² do not fully model human brain pathology in TSC, and thus, analysis of human tuber specimens provides the only direct avenue to study the mechanisms of tuber formation. One strategy to investigate the molecular pathogenesis of cytoarchitectural disorganization in tubers is to evaluate the expression of candidate genes and proteins in human tuber specimens that are relevant to cortical development.¹⁰ Neurotrophins and their cognate receptors comprise a family of proteins that mediate proliferation, differentiation, migration, and process outgrowth during cortical development,^{13,14} and thus, are ideal candidate molecules to investigate in tubers. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4) exert their effects on neurons by binding selectively to a family of neuronal cell membrane receptors, *trks* A to C.^{13,14} NGF signals through *trkA*, BDNF and NT4 through *trkB*, and NT3 through *trkC*. These neurotrophins and their receptors are expressed throughout cortical development and likely contribute to the organized formation of cortical laminae.¹⁵ An additional protein, ciliary neurotrophic factor (CNTF), is enriched primarily in the peripheral

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Table 1. Patient Characteristics

Age/sex	Seizure type	Tuber location	EEG/EcoG	Surgical outcome
Surgical TSC cases				
7/F	cps, gtc	Rt DLF cortex	Rt DLF spikes	Reduced seizures 1 year after surgery
20/F	cps, gtc	Lt Temp cortex	Lt Temp spikes	Seizure free at 2 years after surgery
17/M	cps	Rt Temp cortex	Rt Temp spikes	Reduced seizures at 2 years after surgery
4/M	gtc	Rt DLF cortex	Rt DLF spikes	Reduced seizures at 6.5 years after surgery
1/M	cps	Rt Temp cortex	Rt Temp spikes	Reduced seizures at 4 years after surgery
18/F	gtc	Rt Temp cortex	Rt Temp spikes	Reduced seizures at 3 years after surgery
3/M	gtc, cps	Rt DLF cortex	Rt DLF spikes	Reduced seizures at 4 years after surgery
Post-Mortem TSC cases				
24/F	cps	DLF		
46/F	cps	DLF		

Abbreviations: cps, complex partial seizure; gtc, generalized tonic-clonic seizure; DLF, dorsolateral frontal; Temp, temporal; Rt, right; Lt, left; M, male; F, female; EcoG, electrocorticography.

nervous system and binds selectively to the CNTF receptor (CNTFR).¹⁶ BDNF and NT3 regulate neurogenesis and contribute to differentiation of neuronal progenitor cells within the telencephalic ventricular zone via interaction with trkB and trkC. NT3 and NT4 are critical for dendritic arborization¹⁷ and for axonal pathfinding during corticogenesis. Exposure of developing cortex to excess neurotrophins disrupts cortical lamination *in vitro*¹⁸ and recent evidence suggests that several of these proteins may contribute to epileptogenesis.^{19,20}

Only one study to date has reported expression of trkA and trkB proteins in human cortical dysplasia not associated with TSC²¹ and there has been no investigation of these mRNAs or proteins in TSC. In view of the disorganized cytoarchitecture observed in tubers, we hypothesized that expression of select neurotrophins would be altered in tubers and that these changes may be defined in select cell types. We determined the abundance BDNF, CNTF, CNTFR, NT3, NT4, NGF, trkA, trkB, and trkC mRNAs as well as the chemoattractants netrin1 and netrin2 mRNAs in whole tuber sections resected from TSC patients with medically intractable epilepsy. Netrin1 and netrin2, although not members of neurotrophin family per se, play important roles in axon growth and cell migration, and thus, are also relevant to cortical development.²² We then determined the expression of these mRNAs in single DN and GCs microdissected from tubers in an attempt to define the cellular specificity of these mRNA changes. Although the phenotypic distinctions between these cells remain a source of debate, we attempted to devise a strategy to classify these cells based on size and morphology. Neurotrophin mRNA expression was also evaluated in human NTera2 neurons (NT2N),²³ a human neuronal cell line that expresses p75NGFR, trkA, trkB, and trkC mRNAs.²⁴ These neurons were examined in their native state and after stable transfection with a tuberin antisense construct that reduced tuberin expression. We were specifically interested in

tuberin because overall TSC severity, tuber formation, and epilepsy are likely more severe in TSC2- than TSC1-associated cases.²⁵ Finally, we used Western blotting and quantitative immunohistochemistry with computer-assisted image analysis to demonstrate that observed alterations in neurotrophin mRNA expression predicted changes in protein expression in tubers.

Materials and Methods

Patient Selection and Tissue Samples

Tubers were obtained intraoperatively from seven patients with clinically and radiographically defined TSC²⁶ and medically intractable complex partial epilepsy (Table 1). Tubers were resected from the dorsolateral prefrontal cortex in three patients and the temporal neocortex in four patients. Tubers were identified on preoperative brain MRI and were targeted for surgical resection based on scalp electroencephalography or intracranial electrocorticography that revealed epileptiform discharges such as spikes, sharp waves, or seizures. The histopathological diagnosis was confirmed in these specimens by a neuropathologist (Figure 1). Genotype analysis on four patients was obtained retrospectively and revealed that one case resulted from a mutation in the TSC1 locus whereas three cases resulted from mutations in the TSC2 locus.

Two tubers and adjacent nontuberal dorsolateral frontal cortex was obtained at postmortem examination from two female patients (ages 24 and 46 years) with TSC.²⁶ The postmortem interval to autopsy was 14 ± 4 hours. Both patients had a history of epilepsy. The causes of death were respiratory failure from pulmonary lymphangioleiomyomatosis and myocardial infarction. Histologically, GCs and DN were not present in the nontuberal



Figure 1. Tuber specimen immunolabeled with NeuN antibody. Note disorganized lamination. GCs (**large arrows**) exhibit cytomegaly, a laterally displaced nucleus, and short processes of unclear identity. DNs (**small arrows**) also exhibit a dysmorphic cell soma and disorganized orientation with respect to the pial surface (**top**). Scale bar, 100 μ m.

cortex and the lamination pattern was morphologically similar to control cortex.

Control temporal neocortex was obtained postmortem from four age-matched patients (two females, two males; age, 6 to 36 years; mean age, 22 years). All died of nonneurological causes. Their average postmortem interval to autopsy was 10 ± 3 hours. Seizures were not among the terminal events in these patients and none had a history of epilepsy or TSC. Histological evaluation of these specimens showed no abnormality.

All patients or appropriate family members consented to the use of resected or necropsy material in accordance with the University of Pennsylvania Institutional Review Board and Committee on Human Research.

Tissue Processing and Immunohistochemistry

Brain samples were either fixed by immersion in ice-cold 70% ethanol/150 mmol/L NaCl or 4% paraformaldehyde because previous work²⁷ suggested that rapid cold fixation of human brain specimens yields consistent immunohistochemical results with neurotrophin antibodies. Postmortem specimens were flash-frozen on dry ice at -70°C . Fixed specimens were embedded in paraffin, sectioned at 7 μ m, and mounted on coated slides.

Fixed sections were rehydrated through xylenes and graded ethanols. Sections were immersed in a 150-ml methanol/30 ml H_2O_2 (30%) solution for 30 minutes and then rinsed in cold tap water for 10 minutes. Sections were washed in a 0.1 mol/L Tris (pH 7.4) solution for 5 minutes and then in a Tris/2% fetal bovine serum solution for another 5 minutes. All tuber and control sections were probed with one of several antibodies. In the first experiments, sections were labeled with a mouse monoclonal antibody that recognizes the DNA binding protein NeuN (1:500 dilution; Chemicon, Temecula, CA), a pan-neuronal marker²⁸ that permits reliable identification of neurons in fixed, paraffin-embedded sections before microdissection (see below). In additional experiments, rabbit polyclonal antibodies recognizing BDNF (1:100 dilution),

NT3 (1:100), NT4 (1:100; Santa Cruz Biotechnology, Burlingame, CA), *trkB* (1:50; Chemicon), or *trkC* (1:50; Oncogene, Cambridge, MA) were used to probe tuber and control sections. Primary antibody labeling for all antibodies was performed overnight at 4°C . Immunolabeling was visualized using the avidin-biotin conjugation method (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine. NeuN-labeled sections were stored in 2 \times standard saline citrate buffer awaiting single cell microdissection while the neurotrophin-labeled sections were mounted with Permount (Fisher Scientific, Pittsburgh, PA) for morphometric and densitometric analysis.

In Situ Transcription

The synthesis of a radiolabeled mRNA to probe cDNA arrays begins with generation of cDNA directly on the fixed tissue section by *in situ* (reverse) transcription.²⁹ After NeuN immunolabeling, tissue sections selected for mRNA analysis were treated with Proteinase K (50 $\mu\text{g}/\text{ml}$) at 37°C for 30 minutes and then washed in diethyl pyrocarbonate-treated water. To initiate *in situ* transcription, an oligo-dT₂₄ primer coupled to a T7 RNA polymerase promoter was annealed to cellular poly(A) mRNA directly on the tissue section overnight at room temperature. Sections were then washed in 2 \times standard saline citrate buffer and cDNA was synthesized on the section in *in situ* transcription reaction buffer (10 mmol/L HEPES buffer, pH 7.4, 120 mmol/L KCl, 1 mmol/L MgCl_2 , 250 $\mu\text{mol}/\text{L}$ dATP, dCTP, dGTP, TTP) with avian myeloblastosis reverse transcriptase (0.5 U/ μl ; Seikagaku America, Falmouth, MA). cDNA generated from cellular poly(A) mRNA was extracted from sections with NaOH, 0.1% sodium dodecyl sulfate, and 5 mol/L KAc followed by ethanol precipitation. cDNA from the whole sections was later processed for mRNA amplification and synthesis of a radiolabeled RNA probe.²⁹

NT2N Neuronal Cultures

The NTERA 2 cell line is available through the American Type Culture Collection (NTERA 2 C1.D1, ATCC CRL-1973; Rockville, MD). NTERA 2 human teratocarcinoma cells were differentiated into postmitotic neurons (NT2N) as previously described.²³ Differentiation was induced by culturing cells (3.2×10^3 cells/ cm^2) in the presence of 10 $\mu\text{mol}/\text{L}$ all-trans retinoic acid for 5 weeks followed by replating at 1:6 density without retinoic acid in the presence of mitotic inhibitors (10 $\mu\text{mol}/\text{L}$ 5-fluoro-2'-deoxyuridine, 10 $\mu\text{mol}/\text{L}$ uridine, 1 $\mu\text{mol}/\text{L}$ cytosine- β -D-arabinoside). One week later, a final replating was done using culture vessels coated with Matrigel (Collaborative Biomedical Products, Bedford, MA), and cells were maintained in the presence of mitotic inhibitors for an additional 2 to 3 weeks. The differentiated cultures contain >95% postmitotic neurons, with <5% remaining undifferentiated cells as previously demonstrated by immunofluorescent labeling with anti-MAP-2 antibodies.²³

Generation of Antisense Constructs and Establishment of Transfected NT2N Lines

Three distinct tuberin constructs were generated and subcloned in either the sense (S) or antisense (AS) direction into pcDNA3.1 plasmid vector following a CMV promoter. One construct (S') contains nucleotides 1 to 606 of the full-length tuberin molecule whereas the M and G (GAP domain) constructs contain nucleotides 1902 to 2592 and 4351 to 5347, respectively. The efficacy of the antisense constructs in reducing tuberin expression was first assayed in C6 rat glioma cells 48 hours after lipofectamine-mediated transfection by Western analysis (see below) using rabbit polyclonal tuberin (1:1000 dilution, C-20; Santa Cruz Biotechnology) or mouse monoclonal tubulin (1:5000 dilution, DM1-A; Sigma Chemical Co., St. Louis, MO).

Undifferentiated NT2 cells (4×10^5 cells) were transfected (Calcium Phosphate Transfection Kit; 5 prime->3 prime, Inc., Boulder, CO) with 10 μ g of S- or AS-tuberin expression plasmids for 16 hours, followed by incubation of cultures in medium containing 300 μ g/ml active G418 (Life Technologies, Inc., Gaithersburg, MD). After 10 days, control cells transfected with an irrelevant plasmid had no surviving cells, whereas transfected cell wells showed resistant colonies. NT2N were kept under selection for at least 6 weeks before utilization, to ensure establishment of stable transfectants. These cells were then differentiated into postmitotic neurons (see above).

Microdissection and Aspiration of Single Neurons from Human Specimens and NT2N

The phenotypic distinctions between GCs and DNPs in tubers is a source of debate and indeed, they may reflect a continuum of cell types rather than clearly separate neurobiological cell categories because there are no well-characterized markers to identify either cell type. However, because GCs and DNPs do exhibit morphological and pharmacological differences,^{1,2,9,10} we attempted to more fully evaluate neurotrophin gene expression changes in these cells. For the purposes of single-cell gene and protein expression analysis, GCs and DNPs were defined using morphometric parameters (cell diameter, length, and width) to divide them into groups for analysis. Specifically, using computer-assisted image analysis, we determined the area of >20,000 individual labeled cell bodies in nine tuber specimens. The diameter of the GCs exceeded 80 μ m and GCs exhibited little polarization into axonal or dendritic segments. DNPs were smaller (40 to 70 μ m) and exhibited dendrites and axons that were characteristic of neurons. DNPs also exhibited disorganized orientation with respect to the pial surface. Astrocytes were small (<30 μ m in size) and were excluded from the groups. Cellular morphology was visually corroborated with somatic area and parceled cells into three broad groups. Cells whose area was among the largest 20% of recorded size and that exhibited the most aberrant cell

morphology were deemed GCs. The next largest cell types (the second 20% in cell area size) exhibited features more consistent with neurons and were deemed DNPs. These strict criteria may have actually excluded specific cell types, for example, excessively large DNPs or more diminutive GCs, yet they provided a point of reference to initiate these types of analyses. Before final assignment into GC or DN groups, each 4mm² region of interest (ROI) was visually inspected and any cellular elements erroneously included in the computerized analysis were deleted. In the control sections, all neurotrophin immunoreactive neurons within the targeted 4-mm² ROIs were selected for density analysis. Axons, dendrites, or blood vessels in tuber and control specimens were excluded using maximum-minimum length, width, and area parameters.

Single NeuN-immunolabeled GCs, DNPs, or control pyramidal neurons selected from cerebral cortical layer V were microdissected ($n = 30$ cells in each group) from the sections under light microscopy using a glass Femtotip (Eppendorf) and joystick micromanipulator. Dissected cells were then aspirated into a second glass microelectrode filled with *in situ* transcription reaction buffer and avian myeloblastosis reverse transcriptase. NT2N were fixed briefly in 4% paraformaldehyde before aspiration. Single nontransfected control, S- or AS-transfected NT2N were visualized under phase contrast microscopy and were directly aspirated into glass electrodes filled with *in situ* transcription reaction buffer and avian myeloblastosis reverse transcriptase ($n = 20$ cells in each group). The aspirated cell and reaction buffer were transferred to a microfuge tube and incubated at 40°C for 90 minutes to ensure cDNA synthesis in the single dissected cell.

mRNA Amplification: Tissue Sections, Single Immunolabeled Cells, and Single NT2N

Amplification of mRNA from whole sections and fixed neurons has been described previously in detail.^{10,29,30} cDNA extracted from whole sections or generated from single cells served as a template for synthesis of double stranded template cDNA with T4 DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN). mRNA was amplified (aRNA) from the double-stranded cDNA template with T7 RNA polymerase (Epicentre Technologies). aRNA served as a template for a second round of cDNA synthesis with avian myeloblastosis reverse transcriptase, dNTPs, and N-6 random hexamers (Boehringer-Mannheim). cDNA generated from aRNA was made double-stranded and served as template for a second aRNA amplification incorporating ³²PCTP. The size range of radiolabeled aRNA from whole sections or single GCs and DNPs was assayed on a 1% agarose denaturing gel (not shown). Radiolabeled aRNA from whole tissue sections or single cells was used to probe candidate cDNA arrays.

cDNA Array Analysis

Linearized plasmid cDNAs including BDNF, CNTF, CNTFR (courtesy S. Scherer), NGF, netrin1, netrin2 (courtesy M. Tessier-Lavigne), NT3, NT4, trkA, trkB, and trkC (courtesy R. Madison, Duke University) were adhered to nylon membranes via UV crosslinking to generate a slot-blot array. β -actin, GFAP, and α -internexin cDNAs were included to serve as housekeeping genes to confirm the blot hybridization efficacy. A cDNA encoding tuberin (courtesy J. Sampson, University of Wales, College of Medicine) was also included on the blot so that tuberin mRNA expression could be quantified in each case. Plasmid pBluescript cDNA (pBS) served to define background, nonspecific hybridization of the aRNA probe to the cDNA blot. Blots were hybridized with the radiolabeled aRNA probe for 48 hours in 6 \times SSPE buffer, 5 \times Denhardt's solution, 50% formamide, 0.1% sodium dodecyl sulfate, and 200 μ g/ml salmon sperm DNA at 42°C. Slot blots were washed in 1 \times standard saline citrate and were exposed to a phosphorimager cassette screen for 24 to 48 hours.

Western Analysis

BDNF, NT3, NT4, trkB, and trkC expression in human brain tissue samples and tuberin expression in NT2N were assessed by Western analysis. Frozen tuber and nontuber samples were dissected on a freezing table and homogenized. Tissue homogenates or NT2N were lysed in the RIPA buffer containing 1 \times phosphate-buffered saline, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10% protease inhibitor cocktail (Sigma Chemical Co.). The total cell lysates were centrifuged at 10,000 \times *g* for 15 minutes and the protein concentration estimated in the supernatant using the bicinchoninic acid protein assay (Bio-Rad, Richmond, CA). Cell lysate (100 to 200 μ g) was electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membrane. Western blots were probed with primary antibody (NT3, 1:500 dilution; NT4, 1:500 dilution; trkC, 1:80 dilution; rabbit antituberin, C-20, 1:100 dilution) overnight at 4°C, then with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000 dilution; Amersham, Piscataway, NJ) for 1 hour at room temperature. Antibody labeling was visualized by enhanced chemiluminescence.

Quantitative Immunohistochemical Analysis

Three representative contiguous digital photos were obtained (magnification, \times 20) from each tissue section using image acquisition and analysis software (Phase 3 Imaging System integrated with Image Pro Plus; Media Cybernetics, Silver Spring, MD) and a Spot RT CCD camera (Diagnostic Instruments Inc.).

The relative optical density ratio (ODR) of GCs, DN, and control neurons immunolabeled with BDNF, NT3, NT4, trkB, and trkC antibodies was calculated using the IPP software. Because fixed brain tissue specimens can

bind antibodies with varying avidity, the ODR obviates these differences by determining the intensity of cell immunoreactivity when compared with the noncellular background. The ODR for the selected cell groups served as an index of immunolabeling intensity in single cells and whole sections. The absolute pixel staining density of selected single cells and the noncellular background (the cells were digitally subtracted from the image) was determined in the ROIs from each case and was assigned a numeric value by IPP ranging from the darkest (0) to the whitest (63,535) pixel. A mean optical density value for single GCs or DN in tubers and pyramidal neurons in control cortex was calculated and expressed as a ratio (ODR) of the mean optical density of the background. The total mean ODR for all cells in nine tubers (GCs and DN combined) was tabulated and compared with control neurons for each neurotrophin antibody. The total mean ODR (\pm SE) of GCs and DN separately *versus* control neurons was also compared so that selective changes in individual cell types could be assessed. Statistical comparison of ODR in these groups was accomplished with a one-way analysis of variance and Fisher's post hoc test accepting $P < 0.001$.

Quantitative mRNA Expression Analysis

The relative abundance of neurotrophin and tuberin mRNAs in whole sections, single GCs and DN, and single NT2N was determined by analyzing the aRNA-cDNA hybridization intensities of the cDNA array phosphorimage (ImageQuant 5.0 software). Nonspecific hybridization to pBS plasmid cDNA was subtracted from hybridization intensity of each aRNA-cDNA. Relative hybridization intensity were determined by first averaging the autoradiographic density of all of the aRNA-cDNA hybrids on the blot and then expressing each as a percentage of the average hybridization intensity of the entire blot.³¹ mRNAs whose hybridization intensity was $>125\%$ of the mean blot hybridization intensity were designated as very high abundance, those $>75\%$ of the mean blot hybridization intensity were high abundance, those between 25 to 75% were medium abundance, and those that were $<25\%$ were of low abundance. Differences in relative abundance were determined using a one-way analysis of variance comparing each cDNA against section or cell type. To control for multiple experimental comparisons, a Bonferroni correction factor was used and significance was confirmed with a Fisher's post hoc test ($P < 0.05$).

Results

Fidelity of mRNA Amplification

aRNA amplification using T7 RNA polymerase proceeds by linear kinetics and therefore aRNA-cDNA hybridization intensity reflects relative abundance of cellular poly(A) mRNAs. The fidelity of aRNA amplification procedure from sections and single cells was verified using several approaches. First, only those whole sections and single cell samples with aRNA attaining 2 kb or more in length

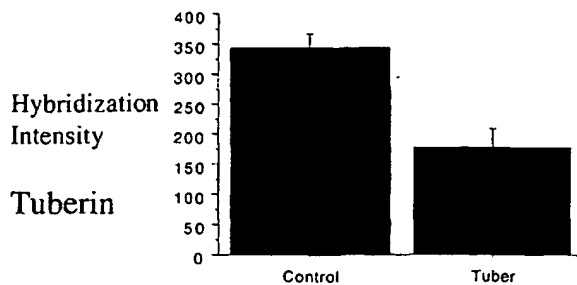


Figure 2. Mean relative hybridization intensity (\pm SE bar) of tuberin mRNA from tuber ($n = 18$) and control ($n = 8$) sections (mRNA amplified from two sections per case). Note overall reduction in tuberin mRNA expression in whole tuber sections compared with control cortex ($P < 0.05$).

as evidenced by a denaturing gel (not shown) were used to probe the arrays. Thus, differences in mRNA abundance on the cDNA arrays did not merely reflect differences in the length of amplified mRNAs from sections and individual cells. Second, the incorporation of 32 P-CTP into aRNA probes was similar in each cell type ($\sim 10^6$ to 10^7 counts incorporated) and thus, differential hybridization of aRNA to the cDNA blots did not reflect variable incorporation of 32 P-CTP into the aRNA probe. Third, aRNA probes from whole sections hybridized to the β -actin, GFAP, and α -internexin cDNAs (not shown) and validated the hybridization conditions. Finally, aRNA probes from sections and single cells exhibited minimal hybridization ($<5\%$ total average blot hybridization intensity) with pBS plasmid cDNA.

Genotype Analysis and Tuberin Expression

In an attempt to define the relationship of neurotrophin gene expression alterations to reductions in tuberin, the abundance of tuberin mRNA was determined in the nine TSC cases. Radiolabeled aRNA from whole tuber sections was used to probe reverse Northern blots containing the tuberin cDNA and hybridization intensity was determined as a measure of mRNA expression. Three cases resulted from mutations at the *TSC2* locus and one case from a *TSC1* locus mutation while genotype information on the five remaining cases was unavailable. Although sample numbers were too few to make appropriate statistical distinctions between cases, by visual inspection, tuberin mRNA expression was similar to con-

trol cortex in the one case associated with a *TSC1* locus mutation (and three other nongenotyped cases). In the three cases associated with a *TSC2* mutation (and the two remaining nongenotyped cases), tuberin mRNA expression was reduced. Indeed, mean tuberin mRNA expression was modestly but significantly reduced (two-fold) across nine all tuber specimens when compared with control cortex (Figure 2).

Differential Expression of Neurotrophin Genes in Whole Tuber Sections

Radiolabeled aRNA probes amplified from tuber, nontuber TSC cortex, and control cortex sections exhibited differential hybridization to the neurotrophin cDNAs. The abundance of neurotrophin mRNAs in control cortical sections was similar to previous reports in normal cerebral cortex as demonstrated by *in situ* hybridization.^{15,32,33} Netrin1 and *trkB* were very high abundance mRNAs; NT3 was a high abundance mRNA; BDNF was a medium abundance mRNA; and CNTF, CNTFR, netrin2, NGF, NGFR, NT4, *trkA*, and *trkC* were low abundance mRNAs in control cortex. The relative expression of these mRNAs did not differ between nontuber cortex and control cortex (percent hybridization when compared to mean hybridization on the blot; Table 2). The assessment of mRNA levels in nontuber cortex compared with control cortex provided a valuable internal control for potentially confounding patient variables in TSC patients such as the effects of longstanding epilepsy, the use of psychotropic and anti-epileptic medications, and the single TSC gene mutation (heterozygote state) presumed present in all nontuber neurons of TSC patients. The similarity in neurotrophin gene expression in nontuber cortex also highlighted the specificity of altered neurotrophin expression within tubers.

When compared with control and nontuber cortex sections, the relative abundance of NT3 and *trkB* mRNAs was reduced in tuber sections (Table 2). In contrast, the relative abundance of NT4 and *trkC* mRNAs was increased in tuber sections. The relative abundance of the chemoattractant netrin1 mRNA was diminished in tuber sections ($301 \pm 26\%$ in controls *versus* $150 \pm 19\%$ in tubers, $P < 0.05$) but netrin2 mRNA was unchanged. The relative abundance of the remaining neurotrophin

Table 2. mRNA Expression and Optical Density Ratios in Tuber and Control Sections

	mRNA expression			ODR section		ODR single cells	
	Control	Tuber	Nontuber	Control	Tuber	GC	DN
BDNF	49 \pm 7%	44 \pm 6%	40 \pm 6%	1.27 \pm 0.01	1.30 \pm 0.02	1.31 \pm 0.02	1.29 \pm 0.02
NT3	70 \pm 8%	33 \pm 7%*	81 \pm 11%	1.38 \pm 0.02	1.27 \pm 0.02*	1.32 \pm 0.01	1.26 \pm 0.03*
NT4	20 \pm 6%	110 \pm 13%*	33 \pm 11%	1.29 \pm 0.02	1.42 \pm 0.01*	1.30 \pm 0.02	1.38 \pm 0.03*
TrkB	187 \pm 30%	103 \pm 12%*	166 \pm 22%	1.35 \pm 0.01	1.29 \pm 0.01*	1.33 \pm 0.03	1.27 \pm 0.02*
TrkC	5 \pm 2%	35 \pm 6%*	7 \pm 1%	1.29 \pm 0.01	1.37 \pm 0.03*	1.49 \pm 0.05*	1.35 \pm 0.03

Left, expression of neurotrophin mRNAs from $n = 12$ control sections (three sections per case), $n = 18$ tuber sections (two sections per case), and $n = 6$ nontuber TSC sections (three sections per case). Numbers reflect summed mean percent hybridization intensity of aRNA amplified from whole tuber sections to neurotrophin cDNA arrays. The hybridization intensity is expressed as a percentage of total blot intensity (*, $P < 0.05$). Middle, relative ODR of neurotrophin-immunolabeled sections reflecting GCs and DNs in tubers and all neurons in control sections (*, $P < 0.01$). Right, relative ODR of GC and DN compared with control neurons in middle panel (*, $P < 0.01$).

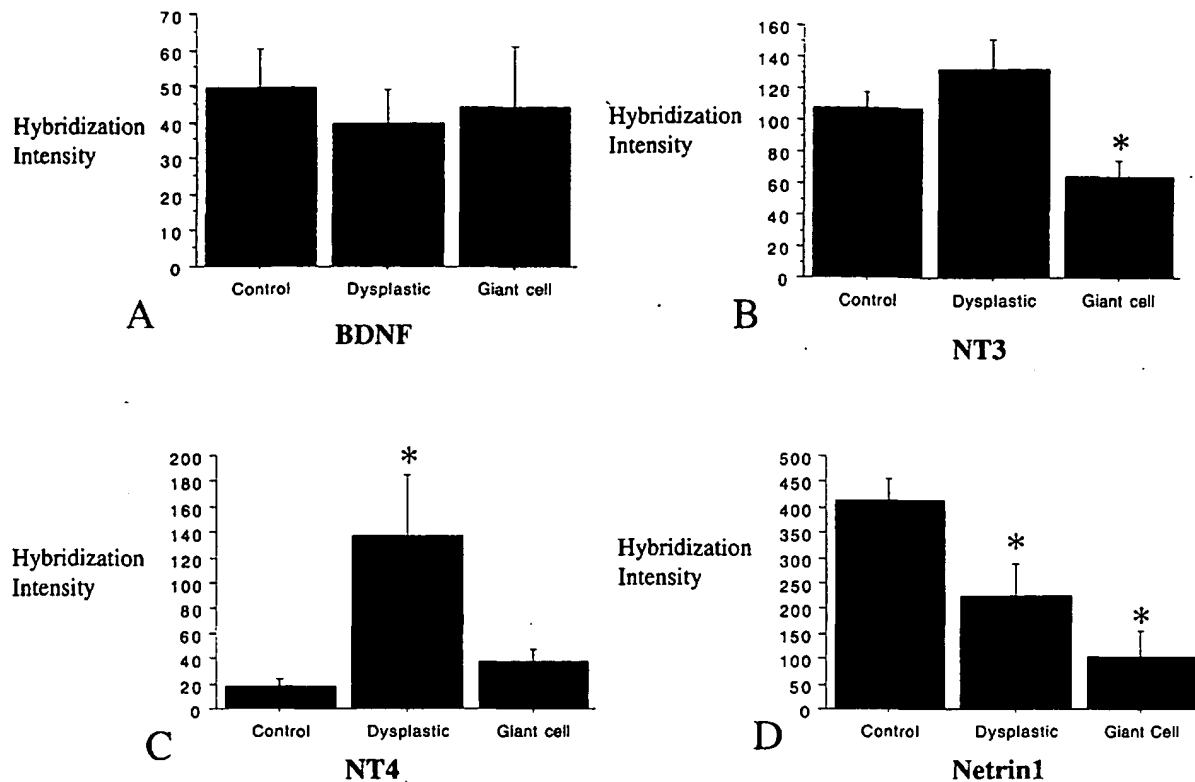


Figure 3. Relative abundance of BDNF, NT3, NT4, and netrin1 mRNAs in single control neurons, DNs, and GCs ($n = 30$ cells in each group; mean percent aRNA-cDNA hybridization intensity, \pm SE bar; *, $P < 0.05$). BDNF mRNA expression was similar in all cell types. Note selective changes in mRNA expression within individual cell types (reduced NT3 mRNA in GCs, increased NT4 mRNA in DNs, and reduced netrin 1 mRNA in both cell types).

mRNAs (NGF, trkA, CNTF, CNTFR) did not differ between tuber, nontuber, and control specimens. Hybridization of aRNA from the entire section to GFAP, α -internexin, and β -actin cDNAs confirmed that representative cellular elements ie, neuronal, glial, endothelial cell types within the tuber contributed to the mRNA expression profile.¹⁰ In an effort to more accurately define alterations in neurotrophin expression solely in DNs and GCs, we assayed mRNA expression in single microdissected cells.

Differential Expression of Neurotrophin mRNAs in GCs and DNs

With the exception of GFAP, all of the assayed mRNAs were detected in control neurons. Netrin1 and trkB were very high abundance mRNAs; NT3 was a high abundance mRNA; BDNF was a medium abundance mRNA; and CNTF, CNTFR, netrin2, NT4, NGF, trkA, and trkC were low abundance mRNAs. Because there were no observed differences in mRNA abundance in nontuber cortex, single cell analysis in these samples was not performed. The detection of α -internexin but not GFAP in the control neurons as well as the DNs and GCs provided strong evidence for the neural phenotype of these cells. The absence of GFAP mRNA in single DNs and GCs allowed us to conclude that amplification of glial mRNAs did not contaminate the cell expression profile data.

The expression of neurotrophin genes was distinct in DNs and GCs when compared with control neurons and highlighted the cellular specificity of the changes in mRNA expression determined in whole sections (Figures 3 and 4). NT4 mRNA expression was increased and trkB mRNA expression was reduced in DNs compared with GCs and control neurons. NT3 mRNA levels were reduced in GCs when compared to DNs and control neurons. In contrast, trkC mRNA abundance was increased and netrin 1 mRNA levels were decreased in both DNs and GCs when compared with control neurons. Expression of BDNF, CNTF, CNTFR, NGF, trkA, and netrin2 mRNAs did not differ across the cell groups.

mRNA Expression in NT2N Transfected with Tuberin Antisense Constructs

Two of the AS constructs (5' and GAP domain) effected a 70% reduction in tuberlin expression in transfected C6 rat glioma cells compared to S-transfected controls. Reduced tuberlin expression in NT2N by Western analysis was most pronounced (~60%) in the NT2N transfected with the 5' AS-construct when compared with nontransfected and S-transfected controls (Figure 5) and thus these cells were assayed for changes in neurotrophin mRNA expression. The expression of control proteins, tubulin, and β -actin (not shown), did not change in the 5'

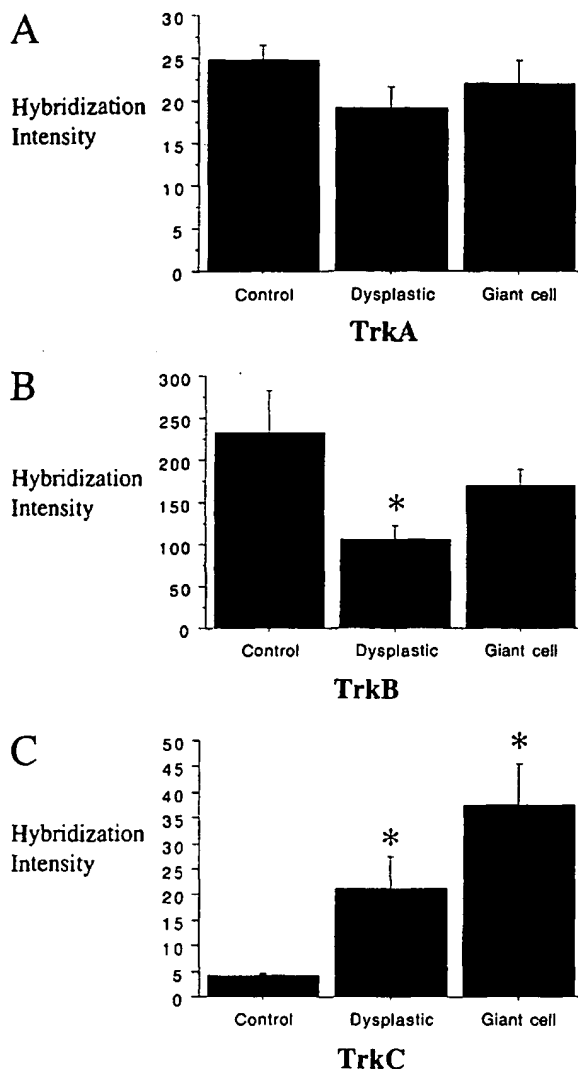


Figure 4. Relative abundance *trkA*, *trkB*, and *trkC* mRNAs in single control neurons, DN, and GCs ($n = 30$ cells in each group; mean percent arNA-cDNA hybridization intensity, \pm SE bar; *, $P < 0.05$). Expression of *trkA* mRNA was similar in all cell types. Expression of *trkB* mRNA was reduced in DN whereas *trkC* expression was increased in both DN and GCs.

AS-transfected C6 glioma cells or the NT2N. Tuberin mRNA levels in the control NT2N and S-transfected NT2N were similar, whereas tuberin mRNA expression was significantly decreased (fivefold reduction) in the AS-transfected NT2N ($n = 20$ cells in each group; Figure 5). No differences in hybridization intensity of α -internexin and β -actin cDNA was observed between the control, S-, and AS-transfected NT2N and GFAP mRNA (an exclusively glial mRNA) was not detected in NT2N. There were no morphological changes observed in the AS-transfected NT2N. Cells extended normal-appearing processes (axons and dendrites) and did not display cytomegaly.

Among the neurotrophin mRNAs analyzed, *trkB* was a very high abundance mRNA; NT3 was a high abundance mRNA; BDNF was a medium abundance mRNA; and CNTF, CNTFR, NT4, NGF, *trkA*, and *trkC* were low abundance mRNAs. The expression of *trkC* and NT4 mRNAs

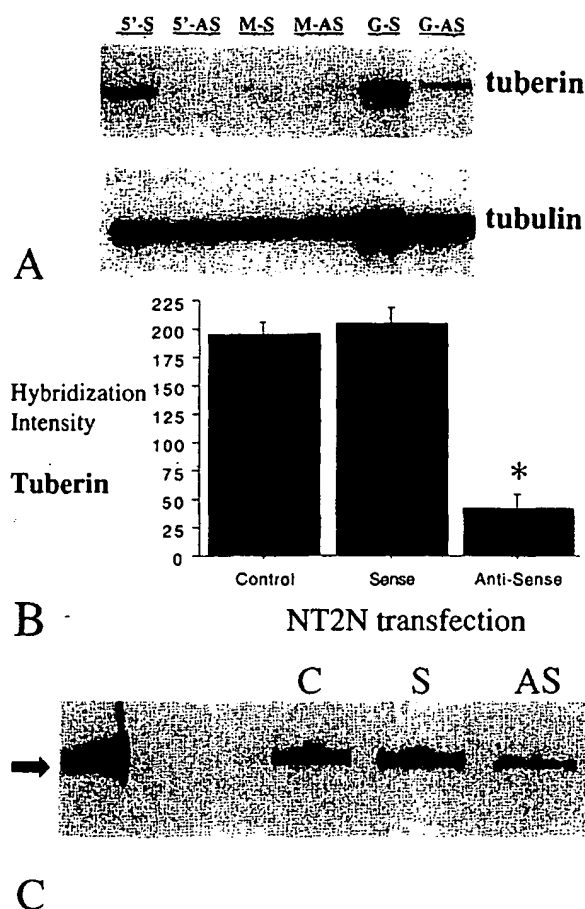


Figure 5. The effect of tuberin antisense constructs on tuberin expression. **A:** Western blot depicting reduced tuberin expression in AS-transfected C6 glioma cells using three distinct AS constructs (5', M-, and G-constructs; see Materials and Methods for nucleotide sequence) compared with S-transfected control cells. No change in control protein tubulin expression in S- or AS-transfected cells. **B:** Relative hybridization abundance of tuberin mRNA in NT2N transfected with 5'-tuberin antisense construct ($n = 20$ NT2N in each group, $P < 0.001$). Note reduction (nearly fivefold) in tuberin mRNA levels in antisense-transfected cells compared with sense-transfected and untransfected control cells. **C:** Western blot depicting reduced tuberin protein expression in NT2N transfected with 5'-tuberin antisense (AS) construct compared with sense (S)-transfected and untransfected control (C) NT2N. Arrow depicts marker size of ~200 kd.

was increased in AS-transfected NT2N compared with nontransfected and S-transfected NT2N (Figure 6). The levels of other neurotrophin mRNAs including *trkB* and NT3 that were altered in tubers, did not differ between control, S-, and AS-transfected NT2N. Netrin1 mRNA was a high abundance mRNA in control and S-transfected NT2N and netrin2 was a medium abundance mRNA. Netrin1 mRNA abundance was reduced in AS-transfected NT2N while netrin2 mRNA levels did not change.

Neurotrophin Proteins in Tubers: Western Analysis and Immunohistochemistry

The expression of select neurotrophin proteins (BDNF, NT3, NT4, *trkB*, and *trkC*) was determined by Western analysis in homogenates of frozen tubers and control cor-

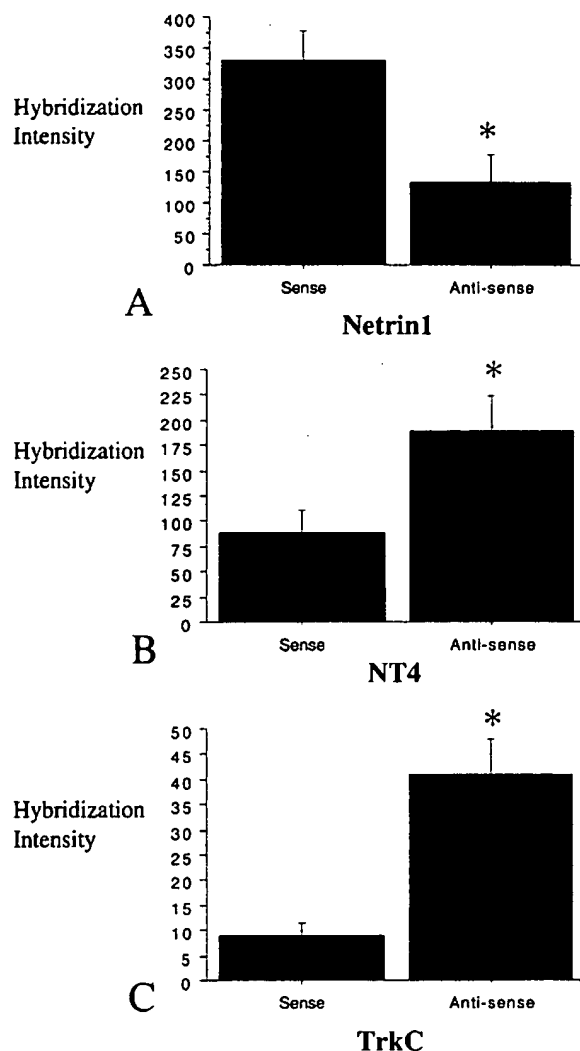


Figure 6. The effect of tuberin antisense constructs on neurotrophin and netrin mRNA expression in NT2N ($n = 20$ NT2N in each group; mean percent aRNA-cDNA hybridization intensity, \pm SE bar; *, $P < 0.05$). **A:** Reduced netrin1 mRNA expression in antisense-transfected NT2N and increased NT4 (**B**) and trkC (**C**) mRNA expression in antisense-transfected NT2N compared with sense-transfected NT2N.

tex. Changes in the abundance of neurotrophin mRNAs identified in whole tuber sections in part predicted altered levels of these proteins in tubers (Figure 7). For example, in two tubers assayed NT4 and trkC protein levels were higher, on visual inspection, than in control cortex. NT3 protein levels were reduced in these two tuber specimens compared with control cortex. BDNF and trkB protein levels did not differ on visual inspection between tubers and control cortex.

Changes in neurotrophin protein expression in whole sections in part reflected altered expression of mRNAs in whole tissue sections. Because differential mRNA abundance in whole tuber sections reflected cell-specific changes in gene transcription as determined by single cell mRNA analysis, we defined the expression of BDNF, NT3, NT4, trkB, and trkC proteins in single GCs and DNs by computer-assisted image quantification of immunohis-

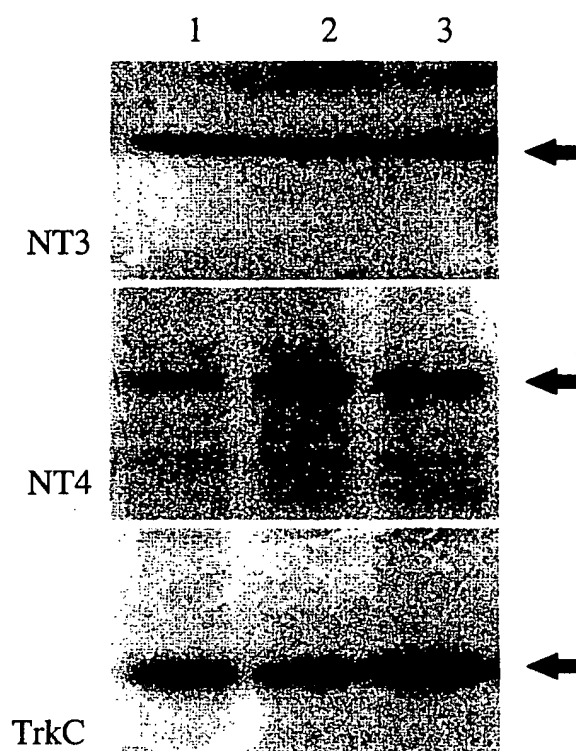


Figure 7. Western analysis depicting altered neurotrophin protein levels (NT3, top; NT4, middle; trkC, bottom) in tuber homogenates. Note reduction in NT3, but increase in NT4 and trkC expression in tubers (lanes 2 and 3) compared with control cortex (lane 1).

tochemically labeled sections. Immunolabeling of nontuber and control cortex with neurotrophin antibodies revealed specific staining patterns consistent with previous reports.^{32,33} For example, robust BDNF and modest NT3 and NT4 labeling was noted in the somatodendritic domains of pyramidal neurons in layers III, V, and VI. BDNF staining was prominent in the soma and apical dendritic segments of pyramidal neurons. In contrast, trkB staining was modest in cells within all cortical layers and faint trkC immunoreactivity was observed in cell bodies and axons of passage throughout the cortical laminae. The patterns of neurotrophin staining in nontuber and control cortex did not differ.

All tuber specimens probed with BDNF, NT3, NT4, trkB, and trkC antibodies exhibited immunolabeling (Figures 8 and 9). Using our morphological criteria for DNs and GCs, the number of neurotrophin immunolabeled GCs and DNs in tubers was determined. A total of 20,053 neurotrophin-immunolabeled cells (GCs and DNs combined for all antibodies) were identified in the nine representative 4-mm ROIs across the nine tuber specimens whereas 6637 neurons were identified in the four control-section ROIs. Within the nine tuber ROIs, 3525 neurotrophin-labeled cells met morphological criteria for GCs whereas 16,528 cells met criteria for DNs demonstrating that the number of DNs far exceeded that of GCs.

The ODR of GCs and DNs combined in tubers and the mean ODR of individual GCs and DNs was determined for each neurotrophin antibody and compared with con-

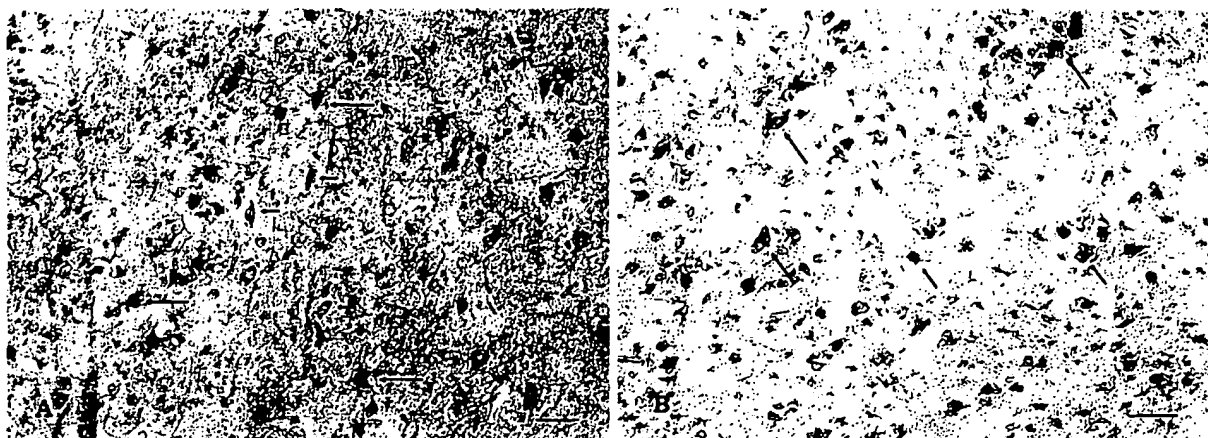


Figure 8. Representative tuber sections probed with neurotrophin antibodies. **A:** trkB-immunolabeled tuber sections. Diminished trkB immunoreactivity in DN neurons (small arrows) compared with GCs (large arrows). **B:** BDNF immunolabeling. Note approximately equal staining density of DN neurons (small arrows) and GCs (large arrows). Scale bar = 150 μ m.

control neurons (Table 2). For example, the NT3 ODR for DN neurons and GCs combined was reduced in tubers compared to control sections and was reduced selectively in DN neurons compared with GCs or control neurons. The NT4 ODR in tubers was increased compared with control sections and the NT4 ODR of DN neurons was selectively increased compared with GCs and control neurons. The trkB ODR was reduced in tubers compared to control cortex and was decreased in DN neurons compared with GCs. The total trkC ODR was increased in tubers versus control sections and was selectively increased in DN neurons and GCs compared to control neurons. BDNF ODR did not differ in tubers versus control cortex or between GCs, DN neurons, and control neurons.

Discussion

We show that expression of select neurotrophin and neurotrophin receptor mRNAs and proteins is altered in tubers compared with nontuber cortex in TSC and control cortex. The changes in neurotrophin mRNA expression determined in whole sections was corroborated in part by Western analysis of neurotrophin protein levels in tuber

homogenates or by densitometry of entire immunohistochemically labeled sections. Thus, the expression of NT4 and trkC mRNA and protein was increased whereas the expression of NT3, trkB, and netrin1 was reduced in tubers compared with control cortex. These results argue that enhanced or diminished transcription of neurotrophin genes in tubers is reflected in expression of the cognate proteins. We generated an *in vitro* system using an antisense tuberin construct to assay the effects of reduced tuberin on neurotrophin expression in a human neuronal cell line that corroborated in part the changes in neurotrophin mRNA and protein expression observed in tubers. Finally, quantitative optical densitometric analysis of immunolabeled sections in part corroborated mRNA expression differences in single DN neurons and GCs. These studies are the first to define altered neurotrophin gene and protein expression in TSC.

mRNA Analysis in Tissue Sections and Single Cells in Neurological Disease

mRNA analysis in archival pathological material provides an experimental strategy to assay alterations in mRNA abundance across select brain lesions such as tubers and to define cell-specific changes in gene expression in individual cell types. Some mRNA degradation occurs within archival pathological material yet numerous investigators have confirmed the fidelity of single-cell mRNA amplification from fixed tissue.^{10,29,30,34-37} These reports have demonstrated that the relative abundance, complexity, and size (often attaining 3 kb in size) of mRNA transcripts amplified from fixed tissue is consistent and can be reliably used for cDNA array analysis.

Single-cell mRNA analysis was designed to surmount the fact that interpretation of gene expression data derived from analysis of whole brain regions may be complicated by the presence of multiple distinct cell types within a region that make differential contributions to the observed profile of mRNA expression.^{10,34,35} Single cell mRNA analysis should be viewed as a strategy to screen

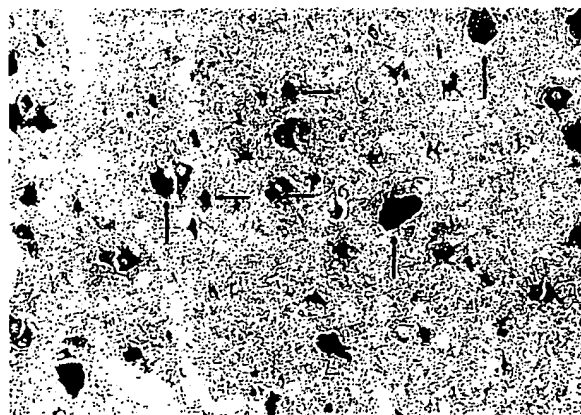


Figure 9. Tuber section probed with trkC antibodies. Note immunoreactivity in both DN neurons (small arrows) and GCs (large arrows).

large numbers of candidate genes that may have significance at the protein expression level. Comparative analysis of gene expression in single cells provides a unique opportunity to identify genes that are differentially expressed within select cell populations. In the present study, single cell mRNA analysis demonstrated that the alterations in gene expression detected in whole sections was explained by differential expression of these mRNAs in distinct cell populations. An important theoretical caveat is that alterations in mRNA levels predict but do not necessarily define alterations in functional protein expression. Additional protein assays such as Western analysis or immunohistochemistry provide corroborative data. For example, altered expression of single GABA_A or NMDA receptor subunit mRNAs was shown to modify neurotransmitter receptor function as defined by patch-clamp recording³⁶ or receptor-ligand binding in whole tissue homogenates.³⁷

Changes in neurotrophin mRNA expression assayed in whole sections and single cells was highly concordant with changes observed in protein expression in tuber homogenates by Western analysis or by densitometric analysis of immunolabeled sections with two exceptions. NT3 mRNA was reduced in whole sections and NT3 protein expression was reduced as determined by Western analysis and densitometry of entire tuber sections. However, in single cells NT3 mRNA was reduced in GCs whereas by single cell immunodensitometry, reduced NT3 protein levels were noted in DN. One explanation is that translational control mechanisms regulate protein expression. Thus, reduced NT3 mRNA in GCs is compensated for by enhanced protein expression and a post-transcriptional modification reduced NT3 protein expression in DN. Similarly, reduced *trkB* mRNA and protein in whole sections and single DN was not detected by Western analysis. The reductions in *trkB* were modest and potentially not detected by Western assay.

Neurotrophin and Tuberin Expression

We wished to specifically investigate the effects of altered tuberin on neurotrophin expression because overall TSC severity, tuber number, and consequently, epilepsy is more severe in *TSC2*- than *TSC1*-associated cases.²⁵ We generated an *in vitro* system using an tuberin AS-construct in human NT2 neurons to reduce tuberin expression because NT2N are a human cell line known to express both hamartin and tuberin.³⁸ The tuberin antisense experiments were designed to model the effects of reduced tuberin, as may occur in gene deletions, as one of many potential mechanisms by which tuberin function may be compromised in patients. Thus, we hypothesized that any observed effects of reduced tuberin expression on neurotrophins in NT2N might corroborate these changes in human neurons *in vivo*. Indeed, NT2N express a variety of neurotrophins²⁴ and the relative abundance of neurotrophin and netrin mRNAs in S-transfected and control NT2N was similar to control cortex. In addition, previous studies in human neuroblastoma cell lines have demonstrated that tuberin AS-constructs reduce tuberin

expression and alter cell cycle dynamics.³⁹ The AS-constructs did not alter cellular morphology in the NT2N, yet tuberin mRNA and protein expression was diminished by >60% and a similar reduction in tuberin expression observed in the C6 glioma cell line supported the validity of the AS-construct effects on tuberin expression. The apparently normal morphology of AS-transfected NT2N cells may have reflected the presence of enough functional tuberin to maintain cellular cytoarchitecture or may require more rigorous evaluation of subtle structural alterations. Increased NT4 and *trkC* mRNA and reduced netrin1 mRNA expression detected in AS-transfected NT2N was similar to that identified in tubers and appeared to be a direct consequence of reduced tuberin levels.

Tuberin mRNA levels were determined in our tissue samples. In five specimens, three of which were obtained from individuals with an identified *TSC2* locus mutation, there was reduced tuberin mRNA levels. In four specimens, one from a patient with a known *TSC1* locus mutation, tuberin mRNA expression was similar to control tissue. Reduced tuberin mRNA levels in two nongenotyped cases supports, but does not prove that these were *TSC2* mutations. In contrast, unchanged tuberin mRNA levels in three of five nongenotyped cases may reflect a *TSC1* mutation but may also support a *TSC2* mutation without quantitative reductions in tuberin gene transcription. In fact, in some TSC tuber specimens, tuberin is robustly expressed in similar populations of cells even in the presence of *TSC2* germline mutations.^{38,40} In our samples, alterations in neurotrophin expression were consistent across all cases and thus we propose that changes in neurotrophin genes and proteins in tubers may result from reduced tuberin function but more likely reflect a common downstream effect of the hamartin-tuberin pathway. There is recent evidence, based on identification of functional protein-protein interactions between hamartin and tuberin, to suggest that hamartin and tuberin comprise a cellular pathway that contributes to cell cycle passage, cell-cell interactions, and possibly cell migration.^{41,42} Taken together, these reports imply that mutations in either the *TSC1* or *TSC2* gene likely results in a downstream cascade of common cellular events that includes changes in neurotrophin expression.

Additional factors may modulate neurotrophin expression in tubers because not all changes in neurotrophin mRNA expression observed in tubers were identified in tuberin AS-treated NT2N. For example, because tuberin reduction in the NT2N was only ~60%, a greater reduction in tuberin may be required to elicit all changes in neurotrophin mRNA expression observed in tuber specimens. Second, tubers consist of a heterogeneous population of cell types whereas the NT2N are a clonal human cell line. Additional effects of astrocytes, GCs, or oligodendrocytes present in human specimens on neurotrophin expression are absent from the NT2N system. Finally, some of the changes in neurotrophin expression may reflect more longstanding developmental effects of *TSC1* or *TSC2* mutations on cells in tubers. Clearly, further studies to define a mechanistic relationship between *TSC1* and *TSC2* gene mutations and changes in neuro-

trophin mRNA expression may prove crucial in understanding tuber formation in TSC.

Altered Neurotrophin Expression and Aberrant Cytoarchitecture in Tubers

NT3/trkC and NT4/trkB constitute two neurotrophin receptor-ligand pathways affected in TSC. If alterations in neurotrophin gene and protein expression persist from embryogenesis, then the downstream effects of these gene expression changes may account for abnormal cytoarchitecture in tubers. The observed changes in gene expression, e.g., enhanced expression of NT4 and diminished expression of trkB, may occur as a direct effect of *TSC1* or *TSC2* gene mutations or imply that changes in the expression of receptor might feedback to diminish expression of its ligand. Recent studies have suggested a role for neurotrophins in the growth and refinement of neural connections,⁴³ dendritic arborization,¹⁷ programmed cell death,⁴⁴ cortical lamination,^{18,45} synaptogenesis,⁴⁶ and in activity-dependent synaptic plasticity.^{47,48} Altered expression of NT3, NT4, trkB, and trkC as well as netrin 1 may have numerous potentially deleterious effects on cortical lamination during development. For example, an intriguing study demonstrated that administration of exogenous NT4 to organotypic cortical slice cultures results in dyslamination and neuronal heterotopia within cortical layers I to II.¹⁷ Thus, enhanced expression NT4 could contribute to the altered laminar cytoarchitecture characteristic of tubers. Reductions in both neuronal and glial cell populations have been observed in NT3 knockout mice.⁴⁹ Overexpression of trkC inhibits the growth of intracerebral xenografts of a medulloblastoma cell line in nude mice.⁴⁴ TrkB knockout mice have decreased densities of axonal varicosities, lower densities of synaptic contacts, and decreased density of synaptic vesicles,⁴⁶ a finding that has been suggested in tubers and non-TSC cortical dysplasia.⁵⁰ Cell culture experiments revealed reduced survival by trkB^{-/-} cortical neurons, a quantitatively significant defect in the formation of dendrites, and a significant reduction in neurite outgrowth by surviving trkB^{-/-} neurons.⁴⁸ Reduced levels of netrin1 are associated with impaired axon pathfinding and aberrant neuronal migration.⁵¹

Neurotrophin Expression, Epilepsy, and Epileptogenesis

Tubers are highly correlated with epilepsy in TSC patients⁵² and presurgical evaluations of TSC patients with medically intractable seizures have demonstrated that tubers are epileptic foci.⁶ Changes in neurotrophin expression in tubers may have relevance to the epileptogenic properties of these lesions. An important theoretical consideration is whether the observed changes in neurotrophin gene and protein expression are secondary to recurrent seizures emanating from the tubers because these specimens were resected for the direct purpose of seizure control. For example, variable increases in NGF,

BDNF, trkB, and trkC mRNA, but decreases in NT3 mRNA expression have been reported after seizures in experimental animals induced by pilocarpine, kainic acid, quinolinic acid, or hippocampal stimulation. Thus, reduced NT3 and enhanced trkC mRNA expression in tubers was consistent with previous experimental seizure paradigms and could in part reflect effects of recurrent seizures on gene transcription. However, NGF and BDNF mRNA expression was not altered in tubers and a decrease (rather than an increase) in trkB mRNA was observed. Thus, a generalized effect of seizures on gene and protein expression in tubers cannot be invoked. Additionally, neurotrophin gene changes in animals are transient and related to early phases of epileptogenesis whereas resected tubers are more chronic epileptic foci. The response of GCs and DNs to seizures may be distinct from normal hippocampal or cortical pyramidal neurons, and thus these neurotrophin gene changes might be unique to tubers.

Altered expression of neurotrophins and trks may contribute to epileptogenesis through a variety of mechanisms. For example, BDNF and NT3 promote the morphological differentiation of a subpopulation of GABAergic neurons in cortex⁵³ and a recent study showed markedly diminished numbers of GABAergic neurons in tubers as evidenced by GAD65 immunolabeling.³⁷ Similarly, NT3 and trkB have a role in the promotion of activity-dependent inhibitory synaptogenesis^{48,49} and thus, reduced NT3 and trkB expression may alter the formation of inhibitory synapses in tubers. Reduced expression of several GABA_A receptor subunits has been demonstrated in tubers and thus, a reduction in GABAergic neurons coupled with diminished GABA-mediated receptor inhibition may foster epileptogenesis. In contrast, a recent study demonstrated that BDNF and NT4 could act as potent excitatory modulators in the hippocampus, cortex, and cerebellum that exerted an effect as rapidly as the neurotransmitter glutamate.²⁰ Thus increased expression of NT4 in tubers could enhance excitatory synaptic activity in these lesions and foster epileptogenesis.

The differential expression of select neurotrophin and chemoattractant genes in tubers suggests pathways that could interfere with appropriate cortical development and promote tuber formation in TSC patients. Further investigation of the functional relationship between these genes and mutations in the TSC genes may shed light on the pathogenesis of tuber formation and epilepsy in TSC patients.

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Expression of brain-derived neurotrophic factor and tyrosine kinase B receptor proteins in glioneuronal tumors from patients with intractable epilepsy: colocalization with *N*-methyl-D-aspartic acid receptor

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Abstract Recent evidence suggests that brain-derived neurotrophic factor (BDNF) and its tyrosine kinase B (TrkB) receptor, in addition to promoting neuronal survival and differentiation, modulates synaptic transmission by increasing *N*-methyl-D-aspartic acid receptor (NMDAR) activity. Overexpression of BDNF may, then, interfere with normal brain function, causing increased excitability. We have examined the immunohistochemical expression of BDNF, full-length TrkB receptor and the NMDAR subunit 1 and subunit 2A/B proteins (NMDAR1 and NMDAR2A/B) in glioneuronal tumors (gangliogliomas, GG, $n=40$; dysembryoplastic neuroepithelial tumors, DNT, $n=15$), from patients with chronic intractable epilepsy. The great majority of tumors studied were positive for all markers examined, supporting the high level of neurochemical differentiation of these lesions. BDNF and TrkB immunoreactivity (ir) was mainly observed in the neuronal component of the tumors. In GG, more than 90% of tumors contained very intense BDNF-ir ganglion cells. Double labeling confirmed the presence of BDNF-ir and TrkB-ir in neurons which contained NMDAR1. NMDAR2A/B intensely labeled abnormal neurons in both GG and DNT and co-localized

with NMDAR1. The presence of BDNF and its receptor in the neuronal component of GG and DNT may suggest a role for this neurotrophin in the development of these lesions, preventing the death of abnormal neuronal cells. In addition, since these neurons contain both NMDAR1 and NMDAR2A/B subunits, the BDNF-TrkB pathway may also contribute through a modulation of glutamatergic transmission to the intrinsic epileptogenicity of glioneuronal tumors.

Keywords Glioneuronal tumors · Brain-derived neurotrophic factor · Tyrosine kinase B receptor · *N*-methyl-D-aspartic acid receptor · Epilepsy

Introduction

Gangliogliomas (GG) and dysembryoplastic neuroepithelial tumors (DNT) are an increasingly recognized cause of chronic intractable epilepsy [18, 72]. Although they may occur throughout the central nervous system (CNS), the temporal lobe is the most common location. Both tumors are composed of a mixture of glial and neuronal elements. This histological composition, which is also a prominent feature of glioneuronal hamartias, has attracted considerable interest with respect to the origin, as well as the intrinsic epileptogenicity of these lesions. Several studies support a maldevelopmental nature of these tumors [26, 54, 68, 78].

Members of the neurotrophin family of growth factors are known to regulate the development and survival of selected nerve cell populations within the peripheral nervous system and CNS [2, 11, 16, 22, 35, 69]. Recently, overexpression of specific neurotrophins has been found to interfere with normal neocortical development [56]. Neurotrophins include nerve growth factor (NGF), neurotrophin-3 (NT-3), NT-4/5, NT-6, and brain-derived neurotrophic factor (BDNF). Of these, BDNF plays a particularly important role in the CNS. BDNF protein and mRNA are broadly distributed throughout the CNS, in contrast with the more restricted expression of NGF, NT-3 or NT-4/5 [10, 12, 17, 20, 32, 47, 52, 70]. BDNF binds to a specific

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receptor with tyrosine kinase activity (the TrkB receptor), which mediates the neurotrophin signaling [29, 30, 44, 66, 79]. The expression of BDNF, as well as TrkB is modulated by neuronal activity under physiological and pathological conditions [8, 41, 43, 49]. In particular, seizure activity has been shown to produce prominent changes in the expression of this neurotrophin and its receptor [3, 21, 24, 47]. Moreover, increasing evidence indicates that BDNF may enhance excitatory synaptic transmission [33, 34, 36, 38, 42, 59]. Studies in different systems, support the involvement of *N*-methyl-D-aspartic acid (NMDA) receptor in BDNF modulation of glutamatergic transmission [27, 37, 39, 57, 62].

In the present study we report the immunohistochemical expression of BDNF, TrkB receptor and specific subunits of NMDAR proteins in glioneuronal lesions associated with intractable epilepsy. Our major aim was to provide data which may help to elucidate the still uncertain origin, development and epileptogenicity of these lesions.

Materials and methods

Subjects

The 55 cases included in this study were obtained from the files of the departments of neuropathology of the Academic Medical Cen-

ter (University of Amsterdam), the Free University in Amsterdam (Prof. Dr. P. v.d. Valk) and the University Medical Center in Utrecht (Dr. G.H. Jansen). Patients underwent resection of GG or DNT for intractable epilepsy. All cases were reviewed independently by two neuropathologists and the diagnosis of GG or DNT was confirmed according to the revised WHO classification of tumors of the nervous system [28]. Of these tumors, 53 (15 DNT and 38 GG) corresponded to grade I; 2 GG were classified as grade II.

The clinical features (derived from patients' medical records) are summarized in Table 1; 40 GG (21 males and 19 females) and 15 DNT (9 males and 6 females) were included in this study. There were 42 temporal lobe tumors (32 GG and 10 DNT). The rest of the tumors ($n=13$) were localized in the frontal lobe. The age at surgery ranged from 6 to 40 (mean 25) years for GG and from 12 to 39 (mean 27) years for DNT. The age at seizure onset ranged from 3 months to 34 years (mean 15 years) for GG and from 1 to 21 years (mean 11 years) for DNT. At the time of surgery the mean duration of epilepsy was 10 years (range 0.6–28 years) for GG and 15 years (range 4–35 years) for DNT. The predominant type of seizure pattern was that of complex partial seizures, which were resistant to maximal doses of antiepileptic drugs (AEDs).

Tissue preparation

Paraffin-embedded tissue was sectioned at 5 μ m and mounted on organosilane (3-aminopropylethoxysilane; Sigma) coated slides. Representative sections of all specimens were processed for hematoxylin/eosin and Nissl stains, as well as for immunocytochemical reactions using a number of neuronal and glial markers described below. Frozen tissue from control and tumor specimens, stored at -80°C , was used for Western blot analysis.

Antibody characterization

Table 2 summarizes the primary antisera used in the present study. Glial fibrillary acidic protein (GFAP), synaptophysin, synapsin, neurofilament protein and neuron-specific enolase (NSE) were used in the routine immunocytochemical analysis of these glioneuronal lesions to document the presence of two distinct populations of tumor cells [9, 58]. The antibodies directed against specific subunits of NMDA receptors have been well characterized in several studies [1, 6, 50, 60, 61, 74, 76, 77]. The antibody to NMDAR1 (mAb 54.1; IgG) was generated against a fusion protein encoding glutathione-

Table 1 Summary of clinical findings of patients with glioneuronal tumors (GG gangliogliomas, DNT dysembryoplastic neuroepithelial tumors)

Type of lesion	Number	Mean age at surgery	Mean age at seizure onset (years; range)	Mean duration of epilepsy
DNT	15	27 (12–39)	11 (1–21)	15 (4–35)
GG	40	25 (6–40)	15 (0.3–34)	10 (0.6–28)

Table 2 Immunocytochemistry: primary antibodies and protocols. Trk (794) is specific for TrkB gp145, and is not cross-reactive with TrkA or TrkC (BDNF brain-derived neurotrophic factor, TrkB tyrosine kinase B receptor, NMDAR *N*-methyl-D-aspartate receptor, NSE neuron-specific enolase, GFAP glial fibrillary acidic protein. RT room temperature)

Antigen	Primary antibody	Source	Microwave treatment	Dilution; technical parameters
BDNF	Polyclonal rabbit	Chemicon (USA)	10 min 650 W	1:50; 16 h, RT
TrkB gp145 (794)	Polyclonal rabbit	Santa Cruz Bio. (USA)	10 min 650 W	1:500; 30 min, RT; 16 h 4°C
NMDAR1	Mouse clone 54.1	[60]	10 min 650 W	1:1,500; 30 min, RT; 16 h, 4°C
NMDAR2 A/B	Polyclonal rabbit	Chemicon (USA)	10 min 650 W	1:100; 30 min, RT; 16 h, 4°C
Synapsin	Mouse clone A 10C	Biotrend (Germany)	10 min 650 W	1:200; 1 h, RT
Synaptophysin	Polyclonal rabbit	Dako (Denmark)	Not required	1:200; 1 h, RT
NSE	Polyclonal rabbit	Sera-Lab (USA)	Not required	1:10,000; 1 h, RT
Neurofilament protein	Polyclonal rabbit	NeoMarkers (UK)	Not required	1:100; 1 h, RT
GFAP	Polyclonal rabbit	Dako (Denmark)	Not required	1:2,000; 30 min, RT; 16 h, 4°C

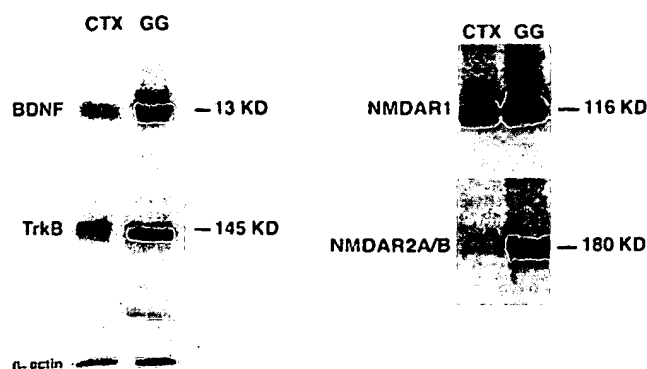


Fig. 1 Expression of BDNF, TrkB, NMDAR1 and NMDAR2A/B in total homogenates from normal temporal cortex and from ganglioglioma, prepared from a patient surgically resected for the treatment of intractable seizures. In the same patient immunocytochemistry revealed a lesion with high neuronal labeling index (>50%) for the neurotrophin, its receptor and NMDAR subunits (see Fig. 2A,C,E,G). Expression of β -actin (as reference protein) is shown in the same protein extracts. Proteins (50 μ g/lane) were subjected to Western blot analysis with specific antibodies (BDNF brain-derived neurotrophic factor, TrkB tyrosine kinase B receptor, NMDAR1, 2A/B N-methyl-D-aspartate receptor 1, 2A/B)

S-transferase in frame with NMDAR1 residues, representing the putative extracellular loop between transmembrane regions III and IV; it recognizes NMDAR1 [60]. The NMDAR2A/B antibody (AB 1548) was raised in rabbit against a 20-amino acid synthetic peptide corresponding to the C terminus of NMDAR2A. It recognizes both NMDAR2A and NMDAR2B [51, 76, 77]. The BDNF polyclonal antibody (AB 1534SP; Chemicon) was raised in rabbit against a 10-amino acid peptide corresponding to the N terminus of mouse BDNF (HSDPARRGEL), which is identical to the corresponding human sequence. The full-length TrkB rabbit polyclonal antibody (TrkB-794, Santa Cruz Bio.) was raised against a peptide corresponding to the amino acids sequence (794–808) mapping adjacent to the C terminus of the precursor form of the mouse TrkB gp145. The specificity of these antibodies was tested by preincubating the antibodies with a 100-fold excess of the antigenic peptides and by Western blots of the total homogenates of human control cortex. BDNF antibody recognized a single band on a Western blot from human material, with molecular mass similar to human recombinant BDNF (13–14 kDa; Fig. 1) and reacted with recombinant BDNF, but not other neurotrophins (NGF, NT-3; data not shown). TrkB recognized a single band of 145 kDa (Fig. 1) and was not cross-reactive with TrkA, TrkC, or the truncated form of TrkB (TrkB gp 95), as previously reported [19].

For immunoblot analysis, tissue from human normal cortex and tumor samples were homogenized in lysis buffer containing 10 mM TRIS pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP40, 5 mM ethylenediamine tetraacetic acid (EDTA) and protease inhibitor cocktail (Boehringer). Protein content was determined using bicinchoninic acid method [63]. Homogenate was diluted to a concentration of 3 mg protein/ml in SDS/bromophenol blue loading buffer, and boiled for 5 min. For electrophoresis, equal amounts of proteins (50 μ g/lane) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 5–20% gels. Separated proteins were transferred to nitrocellulose paper for 1 h, using a semi-dry electroblotting system (Bio Rad, Transblot SD), and incubated in 50 mM TRIS-HCl, 0.1% Tween-20, 154 mM NaCl, pH 7.5 (TTBS), containing 5% non fat dry milk and 1% bovine serum albumin (BSA) for 1 h. Samples were then incubated over night in TTBS/3% BSA/0.1% sodium azide, containing the primary antibody [BDNF 1:500; TrkB 1:5,000; NMDAR1 1:10,000; NMDAR2A/B 1:1,000; monoclonal anti-actin antibody (Sigma, St. Louis, Mo.), 1:1,000]. Af-

ter several washes in TTBS, the membranes were incubated in TTBS/5% non fat dry milk/1% BSA, containing the goat anti-rabbit or anti-mouse coupled to horseradish peroxidase (1:1,500; Dako) for 2 h. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (ECL, Amersham). Because of the limited availability of frozen material for these type of tumors (4 GG, but no DNT), a complete analysis by immunoblot was not possible.

Immunocytochemistry

The sections were deparaffinated in xylene and after rinses in ethanol (100% and 95%) were incubated with 1% H_2O_2 diluted in methanol for 20 min. Slides were then washed with phosphate-buffered saline (PBS; 10 mM, pH 7.4). For BDNF, TrkB, NMDAR1, NMDAR2A/B and synapsin immunocytochemistry, the slides were placed in sodium citrate buffer (0.01 M, pH 6.0) and heated in a microwave oven at 650 W for 10 min, then washed in PBS and incubated with a mixture of 10% normal goat serum (NGS), 0.1% gelatin and 5% BSA for 1 h prior to the incubation with the primary antibody at dilutions, temperatures and duration specified in Table 2. At this point the sections were washed thoroughly with PBS and incubated at room temperature (RT) for 1 h with the appropriate biotinylated secondary antibody diluted in PBS [1:400 goat-anti rabbit immunoglobulin (Ig) or 1:200 goat-anti mouse Ig from Dako]. Single-label immunocytochemistry was carried out using avidin-biotin peroxidase method (Vector Elite) and 3,3'-diaminobenzidine (DAB) as a chromogen. Sections were counterstained with hematoxylin, dehydrated in alcohol and xylene and coverslipped. Sections incubated without the primary antibody or with preimmune sera were essentially blank. Immunocytochemistry performed with BDNF or TrkB antibody preabsorbed with 100-fold excess of the specific peptide showed only a very light background staining. As positive controls for immunohistochemical staining, surgical and autopsy specimens from normal temporal and frontal lobes were used.

Double-label immunocytochemistry was performed by incubating sections with anti-NMDAR1 and anti-NMDAR2A/B (or anti-BDNF, or anti-TrkB) at dilutions, temperatures and duration specified in Table 2. Following three washes in PBS, sections were then incubated for 1 h at RT with goat anti-rabbit Ig conjugate to peroxidase labeled-dextran polymer (Peroxidase rabbit; EnVision⁺, ready to use, Dako) and 1:200 biotinylated goat anti-mouse (gam-BIO; Dako). After washes in PBS, sections were incubated for 45 min with a streptavidin alkaline phosphatase complex (strAP; Dako, 1:100 in PBS). Sections were then washed first with PBS and after with TRIS-HCl buffer (0.1 M; pH 8.5). The first chromogen used was Fast blue B salt (Sigma); 2 mg in 0.1 M TRIS-HCl buffer pH 8.5, containing 3 mg naphthol-AS-MX-phosphoric acid and 1 mM levamisole. After washes, slides were stained with 3-amino-9-ethyl carbazole (AEC; Sigma). Sections were then washed with PBS, rinsed in distilled water, and mounted with glycerin-gelatin (Dako).

Evaluation of immunostaining

Both single- and double-labeled tissue sections, from each type of lesion and immunocytochemical marker, were examined by two observers with respect to the presence of specific immunoreactivity (ir) for the different markers in glial cells and neurons. The immunostaining was rated and a consensus score was obtained. Neurons and glia in the infiltration zone were not included. We rated the degree of staining for all antibodies on a semiquantitative four point scale, where immunoreactivity was defined: –, not present; \pm , moderate; +, intense; and ++, very intense. Moreover, the labeling indices in the neuronal component of the tumor (number of labeled neurons per total number of neurons) for each marker was assigned semiquantitatively to four categories: <1%, 1–10%, 11–50%, >50%.



◀ **Fig. 2** Representative photomicrographs of immunohistochemical staining for BDNF, TrkB, NMDAR1 and NMDAR2A/B in GG (A,C,E,G) and DNT (B,D,F,H). Sections are counterstained with hematoxylin. A,B Immunoreactivity for BDNF. A GG: strong cytoplasmic staining is observed in both small and large atypical neurons (arrows; asterisk shows a binucleate BDNF-positive ganglion cell). B DNT: a BDNF-positive neuron (arrow) is located in a fibrillary tumor matrix. There are also smaller immunoreactive tumor cells (arrowheads). C,D Immunoreactivity for TrkB receptor. C GG: Neuronal cells of different size with membranous and cytoplasmic staining for TrkB (arrows). D DNT with TrkB-positive neurons (arrows) and smaller immunoreactive oligodendrocyte-like cells (arrowheads). E,F Immunoreactivity for the NMDAR subunit 1. E GG: strong NMDAR1-positive large neuron (arrow). F DNT: single mature neuronal cells with strong NMDAR1 immunoreactivity (arrow), surrounded by smaller NMDAR1-positive cells (arrowheads). G,H NMDAR2A/B immunostaining. G GG with a group of NMDAR2A/B-positive neuronal cells (arrows). H NMDAR2A/B-positive neuron (arrow). In addition few oligodendrocyte-like cells are also positive (arrowheads) (GG ganglioglioma DNT dysembryoplastic neuroepithelial tumor). Bar 35 μ m

Results

Case material and histological features

The clinical features of the cases included in this study are summarized in Table 1. There were 40 GG and 15 DNT and all patients had a history of chronic pharmaco-resistant epilepsy. Postoperatively, 24 patients with GG (60%) and 10 patients with DNT (67%) were completely seizure free. The long-term follow up of seizure outcome after surgery in glioneuronal tumors (including that of most of the lesions of the present series) will be presented elsewhere (Aronica et al., in preparation). Histologically, GG were composed of a mixture of neoplastic astrocytes and abnormal neuronal cells and presented a broad spectrum of histopathological features. The glial component consisted mainly of fibrillary astrocytes with different degree of cellularity and strong immunoreactivity to GFAP. The neuronal component, variable in amount, was characterized by cells with lack of uniform orientation, with abnormal shape, vesicular nuclei and prominent nucleoli (Fig. 2A, C, E, G). Binucleate neurons were observed in 31 patients. The size of the ganglion cells was extremely variable. In the same lesion, normal-sized neurons with a scant cytoplasm were found together with giant atypical ganglion cells.

Histologically, DNT had a complex nodular or multinodular intracortical architecture with a typical heterogeneous cellular composition. They contained a complex mixture of neuronal cells, astrocytes and a prominent population of oligodendroglia-like cells (Fig. 2B, D, F, H). NSE-ir cells were demonstrated in all the tumors examined (40 GG and 15 DNT). Immunoreactivity for the neurofilament protein was present in 38 GG and 13 DNT. Intense synapsinir and synaptophysin-ir was detected in 37 GG and 10 DNT. In 11 GG a cytoplasmic staining for synaptophysin was the prominent pattern of immunoreactivity.

BDNF and TrkB receptor expression in glioneuronal tumors

Western blot analysis showed the specificities and protein contents for BDNF and full-length TrkB receptor in homogenates from human temporal cortex and ganglioglioma, prepared from a patient operated for intractable epilepsy. Both BDNF- and TrkB-specific bands (13 kDa and 145 kDa, respectively) of the glioneuronal lesion appeared significantly denser than that of normal cortex from the same patient (Fig. 1). Immunocytochemistry for BDNF and its receptor demonstrated immunoreactivity for both markers in the neuronal component of all GG and DNT examined (Fig. 2; Table 3). BDNF cellular staining appeared as a diffuse reaction product mainly localized in the perikaryal cytoplasm and occasionally extended into the most proximal processes, but not the cell nucleus. The cellular localization of immunoreactivity for the neurotrophin and its receptor was similar to that observed in control specimens and described in the literature in both rat and human brain [12, 48, 64, 71]. However, the two antibodies labeled the atypical neurons more intensely than the normal neuronal cells in both type of lesions.

Figure 2A shows BDNF expression in a representative GG. Both small and large size neurons showed strong cytoplasmic staining. High BDNF-ir was observed in 92% of GG within ganglion cells of various size. Small neuronal cells showed mainly a moderate staining, observed in 75% of the tumors. The labeling index of neuronal cells usually ranged from 11% to 50%, whereas 5 cases had more than 50% labeled cells. Rare (less than 1%) BDNF-positive astrocytes were present in 10% of GG. Figure 2B shows

Table 3 Expression of BDNF, TrkB NMDAR1 and NMDAR2A/B in glioneuronal tumors. Values given as % of cases with immunoreactive cells. Immunoreactivity: – not present; \pm moderate; + intense; ++ very intense

Tumor	BDNF			TrkB			NMDAR1			NMDAR2A/B		
	\pm	+	++	\pm	+	++	\pm	+	++	\pm	+	++
Ganglioglioma (n=40)												
Ganglion cells	3	5	92	7	80	13	8	47	25	23	47	8
Small neuronal cells ^a	17	75	8	27	50		30	38	–	43	18	–
Astrocytes	10	–	–	5	–	–	25	–	–	15	–	–
DNT (n=15)												
Neurons	27	60	13	40	53	7	20	46	7	33	20	13
Oligodendrocyte-like cells	20	13	–	27	33	13	13	33	20	13	20	–
Matrix	67	–	–	–	–	–	40	–	–	27	–	–

^aSmall neuronal cells with a scant cytoplasm

BDNF expression in a representative DNT. In the DNT many neurons (ranging from 11% to 50%) showed cytoplasmic BDNF immunoreactivity, but to a varying degree. High BDNF-ir was observed in only 13% of DNT, whereas moderate immunoreactivity was encountered in the majority of the tumors (60%). Oligodendrocyte-like cells showed low (in 3 cases) to moderate (in 2 cases) immunoreactivity for BDNF. Staining of the tumor matrix was observed in 67% of the cases.

Immunoreactivity for the TrkB receptor protein appeared as membranous/cytoplasmic staining. Figure 2C shows TrkB receptor expression in a representative GG. For TrkB protein many giant neuronal cells, as well as small neurons (ranging from 11% to 50%) showed cytoplasmic staining to a varying degree (Table 3). Moderate staining was found in 80% of the cases in ganglion cells and in 50% of the cases in small neuronal cells. Most of the astrocytes were negative for TrkB. Rare TrkB-positive astrocytes were observed in two cases. Figure 2D shows TrkB receptor expression in a representative DNT. In all DNT examined there were neurons immunoreactive for TrkB and in most of the cases moderate immunoreactivity was observed (53% of the tumors). The labeling indices usually ranged from 11% to 50%. In addition, TrkB-ir was observed in oligodendrocyte-like cells in 11 cases (Table 3).

To detect changes in the expression of BDNF and its receptor in the perilesional zone we examined cases in which both perilesional cortex and normal cortex were available (5 GG and 5 DNT). Immunocytochemistry, however, did not reveal changes of either BDNF or TrkB protein expression in the peritumoral area when compared to normal cortex (data not shown).

NMDAR1 and NMDAR2A/B expression in glioneuronal tumors

Western blot analysis confirmed the specificity of NMDAR1 and NMDAR2A/B in human cortical homogenates. Figure 1 shows specific bands (116 kDa for NMDAR1 and 180 kDa for NMDAR2A/B) identical to that previously described [77]. On blot the NMDAR2A/B band of tumor material was significantly denser than that of normal cortex from the same patient.

The great majority of the lesions had neuronal immunoreactivity for NMDAR1 and NMDAR2A/B proteins. The overall pattern of immunoreactivity was similar to that observed in control specimens and described in the literature [1, 6, 50, 60, 61, 74, 76, 77].

NMDAR1-ir was present within the cytoplasm and at the neuronal membrane. NMDAR1-positive ganglion cells were observed in 80% of GG (Fig. 2 E and Table 3). NMDAR1-ir was also encountered in small neuronal cell in 27 GG (68%). Of the 32 GG with NMDAR1-positive neuronal cells, 4 (12%) showed less than 1% labeled neurons, 9 (28%) had a labeling index between 1% and 10%, 16 (50%) had a labeling index between 11% and 50%, and 3 (9%) had a labeling index greater than 50%. In addition,

astrocytes were found NMDAR1-positive in 10 cases (25%). In DNT neurons immunoreactive for NMDAR1 were also frequently encountered (Fig. 2F and Table 3). Of the DNT, 73% contained NMDAR1-ir neurons with labeling indices usually ranging from 11% to 50%, whereas 4 cases had more than 50% labeled neuronal cells. Oligodendroglia-like cells were found immunoreactive for NMDAR1 in 10 cases (66%). NMDAR1-ir was also localized in the fibrillar glial matrix in 40% of the tumors.

In contrast to the control cortex from autopsy, as well as surgical specimens, which showed no or little detectable NMDAR2A/B-ir (data not shown), the expression of NMDAR2A/B protein was frequently encountered in the neuronal component of GG (Fig. 2G; Table 3). NMDAR2A/B-ir was observed in 78% of GG. Many giant neurons (labeling indices usually ranged from 11% to 50%) showed NMDAR2A/B staining distributed throughout the cell body and the dendritic processes. Of the GG, only 6 contained astrocytes with slight positivity for NMDAR2A/B. In DNT, neurons immunoreactive for NMDAR2A/B were found in 60% of the cases, with labeling indices ranging from 11% to 50% (Fig. 2H; Table 3). Neuronal cell bodies in DNT were stained to a varying degree, the nuclei were clear, and NMDAR2A/B-positive dendrites were observed. In 33% of the DNT, a subpopulation of the oligodendroglia-like cells showed also NMDAR2A/B-ir. NMDAR2A/B-ir localized in the fibrillar matrix in 27% of the cases.

It is known that NMDAR1 forms a functional receptor complex subunit only when it is expressed in heteromeric combinations with the NMDAR2 subunits [45, 65]. Figure 3A shows a representative GG in which clusters of NMDAR2-positive neuronal cells also express NMDAR1 subunit. NMDAR1 co-localized with NMDAR2A/B also in the neuronal component of DNT (data not shown).

Colocalization BDNF/TrkB/NMDAR1

Several studies indicate the NMDA receptor as key target of the action of BDNF in modulating the glutamatergic transmission [14, 27, 37, 39, 57, 62]. To determine if NMDAR1-positive neurons also contain BDNF and its receptor we performed a double labeling with BDNF or TrkB antibodies. Sections of 6 GG and 6 DNT, which contained high neuronal labeling indices for the NMDAR1 and BDNF were used for the double-labeling studies. Figure 3B shows a representative example of a GG, where ganglion cells are both positive for NMDAR1 and BDNF. The NMDAR1-ir is more densely distributed at the cell membrane, whereas BDNF-ir is cytoplasmic. NMDAR1-ir ganglion cells were found to express also the TrkB receptor protein. Double-labeling with NMDAR1 and BDNF or TrkB antibodies demonstrated colocalization of these markers also in the neuronal component of DNT (data not shown).

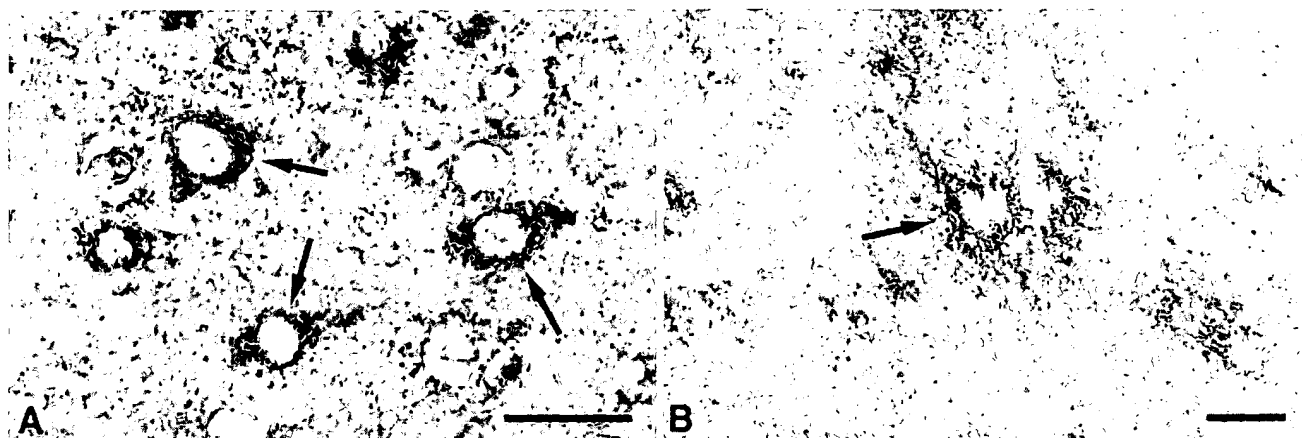


Fig. 3 Colocalization of NMDAR1 with NMDAR2A/B or BDNF in the neuronal component of ganglioglioma. **A** A group of neuronal cells in GG (arrows) stained in purple as result of the colocalization of NMDAR1 (blue) with NMDAR2A/B (red). **B** Colocalization in ganglion cells (arrows) of BDNF (cytoplasmic red staining) with NMDAR1 (membranous blue staining). Bars **A** 45 μ m; **B** 35 μ m

Discussion

Both gangliogliomas and dysembryoplastic epithelial tumors are frequently associated with chronic epilepsy [18, 72]. A common histopathological feature of these lesions is represented by the presence of highly differentiated neuronal cells, which can actively participate in interneuronal signal transduction [74, 75]. In the present study we report high expression of BDNF and its receptor in the neuronal component of glioneuronal tumors. We also show that aberrant BDNF/TrkB-positive neurons express both NMDAR1 and NMDAR2 subunit proteins. The significance of these findings is discussed below.

BDNF/TrkB/NMDAR expression and histogenesis of glioneuronal tumors

The histogenesis of glioneuronal tumors remains still speculative; however, one possible hypothesis is their origin from still immature or multipotent dysplastic cells. In support of the malformative nature of these tumors is their common association with dysplastic/malformative abnormalities of the cortical architecture [15, 26, 53, 54, 55, 73]. Moreover, a stem cell marker CD34 has been recently observed within the majority of gangliogliomas and glioneuronal hamartomas, suggesting a common origin from a multipotent precursor [5].

BDNF, acting through its receptor TrkB, is known to influence the fate of multipotent cells early during development [2, 40]. While in physiological conditions abnormal dysplastic neurons undergo cell death via apoptosis, abnormal neurons which co-express BDNF and TrkB may escape this selection and develop into a glioneuronal lesion. Anti-BDNF antiserum reduces the survival of cultured

cortical neurons that co-express BDNF and TrkB mRNA [23]. Moreover, TrkB receptor expression has been recently observed in dysplastic neurons within foci of cortical dysplasia [48] and the overexpression of BDNF in developing brain has been related to the induction of different types of malformations, including heterotopias and aberrant cortical lamination [56]. The BDNF/TrkB pathway could, then, represent the link between early developmental disorders and glioneuronal tumors.

In our series BDNF and TrkB proteins were mainly expressed in the neuronal component of the tumor (ganglion and small neuronal cells), suggesting the existence of a BDNF autocrine loop in these cells. Whereas only few BDNF- or TrkB-positive astrocytes were detected, the more frequent presence of BDNF-ir and TrkB-ir in oligodendroglia-like cells suggests an advanced grade of neuronal maturation of these cells. In agreement with previous reports [75], oligodendroglia-like cells in DNT express NMDAR subunits, as additional markers of neuronal differentiation. Although further studies are needed to assess the expression of BDNF and its receptor in more immature tumors, the BDNF/TrkB pathway may be critical with respect to the survival, as well as the differentiation of neuronal cell tumors. Accordingly, the expression of neurotrophins and their receptors has been shown to be closely correlated with the differentiation state of the tumor cells in different types of CNS tumors [7, 25, 46, 48, 67].

Since the glioneuronal tumors examined were all associated with epilepsy, which has been shown to regulate the expression of BDNF and its receptor [3, 21, 24, 47], we can not exclude that chronic seizure activity could contribute to the strong BDNF and TrkB expression observed in the neuronal component of the tumors.

BDNF/TrkB/NMDAR expression and epileptogenicity of glioneuronal tumors

The expression of BDNF and its receptor in the neuronal component of GG and DNT may also help to understand the development and maintenance of epileptic activity in these tumors. The role of BDNF in epileptogenesis is suggested by the marked delay of seizure development ob-

served in BDNF mutant mice [31] or induced by intraventricular administration of TrkB "receptor bodies", which selectively prevent trkB activation [4]. Transgenic mice overexpressing BDNF show hyperexcitability and increased seizure severity [13]. In addition, several studies demonstrate that activity-dependent up-regulation of BDNF levels may in turn increase synaptic transmission via modulation of postsynaptic NMDA receptors. This is due, at least in part, to phosphorylation of specific NMDAR subunits, mediated by TrkB activation [37, 39].

In the present study we demonstrate that atypical neurons in glioneuronal tumors express both NMDAR1 and NMDAR2 subunit proteins. The subunit composition of the NMDAR is critical for its physiological action. In particular, the NMDAR2 subunits define the functional properties of the native NMDA receptor. Whereas NMDAR2 subunits alone show no responses to glutamate, in heteromeric combination with NMDAR1 produce currents 20 to 60 times larger than homomeric NMDAR1 channels [45, 65]. Thus, the colocalization of both NMDAR1 and NMDAR2A/B in the neuronal component of GG and DNT supports the potential hyperexcitability of these cells, which could contribute to focal seizure generation. Interestingly, selective coexpression of NMDAR2A/B and NMDAR1 subunit proteins has also been recently described in aberrant neurons in epilepsy-associated cortical dysplasias [76, 77].

Another crucial question relevant to the epileptogenesis in these lesions is whether NMDAR-expressing neurons produce BDNF and possess the TrkB receptor proteins able to mediate the neurotrophin actions. In the present study, we clearly demonstrate that NMDAR1-positive neurons contain BDNF and express its receptor TrkB. Thus, BDNF through its receptor may potentially increase NMDAR activity in the neuronal component of the tumors.

The data presented in this study demonstrate the presence in glioneuronal tumors of a population of probably hyperexcitable cells (expressing heteromeric NMDAR1-NMDAR2 channels), in which the BDNF/TrkB pathway, may play a critical role in the development, as well as in the epileptogenic mechanisms of these lesions. Future studies to further define the role of the BDNF/TrkB pathway in epileptogenesis will be important for the development of new targeted drugs, which could improve the treatment of epilepsy-associated focal lesions.

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7) **Expression and function of TRK-B and BDNF in human neuroblastomas.**

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Molecular and cellular biology (UNITED STATES) Jan 1994 , 14 (1)
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8) ****Neurotrophins and Trk receptors in human pancreatic ductal adenocarcinoma: expression patterns and effects on in vitro invasive behavior.**

Miknyoczki S J; Lang D; Huang L; Klein-Szanto A J; Dionne C A; Ruggeri B
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International journal of cancer. Journal international du cancer (UNITED
STATES) May 5 1999 , 81 (3) p417-27, ISSN 0020-7136--Print
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9) **Differential cellular expression of neurotrophins in cortical tubers of the tuberous sclerosis complex.**

Kyin R; Hua Y; Baybis M; Scheithauer B; Kolson D; Uhlmann E; Gutmann D;
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10) **Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina.**

Ghazi-Nouri S M S; Assi A; Limb G A; Scott R A H; von Bussmann K;
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British journal of ophthalmology (England) Apr 2003 , 87 (4)
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11) **Expression of NGF family and their receptors in gastric carcinoma: a cDNA microarray study.**

Du Jian-Jun; Dou Ke-Feng; Peng Shu-You; Qian Bing-Zhi; Xiao Hua-Sheng;
Liu Feng; Wang Wei-Zhong; Guan Wen-Xian; Gao Zhi-Qing; Liu Ying-Bin; Han
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World journal of gastroenterology - WJG (China) Jul 2003 , 9 (7)
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12) **ssociation of neurotrophin receptor expression and differentiation in human neuroblastoma.**

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13) **Expression of brain-derived neurotrophic factor and tyrosine kinase B receptor proteins in glioneuronal tumors from patients with intractable epilepsy: colocalization with N-methyl-D-aspartic acid receptor.**

Aronica E; Leenstra S; Jansen G H; van Veelen C W; Yankaya B; Troost D

Expression and Function of *TRK-B* and *BDNF* in Human Neuroblastomas

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There is considerable interest in the role of the *TRK* family of neurotrophin receptors in regulating growth and differentiation in normal and neoplastic nerve cells. A neuroblastoma is a common pediatric tumor derived from the neural crest, and the majority of favorable neuroblastomas express a high level of *TRK-A* mRNA. However, little is known about the expression or function of *TRK-B* in these tumors. *TRK-B* encodes a tyrosine kinase that binds to brain-derived neurotrophic factor (BDNF), as well as neurotrophin-3 (NT-3) and NT-4/5. We have studied the *N-myc*-amplified human neuroblastoma cell line, SMS-KCN, which expresses both *TRK-B* and *BDNF*. Exogenous BDNF induces tyrosine phosphorylation of *TRK-B* as well as phosphorylation of phospholipase C- γ 1, the extracellular signal-regulated kinases 1 and 2, and phosphatidylinositol-3 kinase. BDNF also induces expression of the immediate-early genes *c-FOS* and *NGFI-A* but not *NGFI-B* or *NGFI-C*. In addition, BDNF appears to promote cell survival and neurite outgrowth. SMS-KCN cells also express *TRK-A*, which is phosphorylated in response to nerve growth factor. However, the downstream *TRK-A* signaling is apparently defective. Finally, we determined that in a series of 74 primary neuroblastomas, 36% express *TRK-B* mRNA, 68% express *BDNF* mRNA, and 31% express both. Truncated *TRK-B* appears to be preferentially expressed in more-differentiated tumors (ganglioneuromas and ganglioneuroblastomas), whereas full-length *TRK-B* is expressed almost exclusively in immature neuroblastomas with *N-myc* amplification. Our findings suggest that in *TRK-B*-expressing human neuroblastomas, BDNF promotes survival and induces neurite outgrowth in an autocrine or paracrine manner. The BDNF/*TRK-B* pathway may be particularly important for growth and differentiation of neuroblastomas with *N-myc* amplification.

Developing neurons require trophic factors for survival, growth, and differentiation (4, 20). Nerve growth factor (NGF) was first discovered as a neurotrophic factor that supports the development and maintenance of peripheral sympathetic and neural crest-derived sensory neurons (35, 63). NGF is a member of a family of proteins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) (14, 23, 25, 38, 56), and NT-4/5 (7, 19). Despite the high sequence homology, the developmental and physiological functions of each factor appear to be different.

Recently, three receptors for the neurotrophic factors of the NGF family have been cloned, and they also form a gene family encoding protein tyrosine kinases (28, 32, 33, 41-43). *TRK-A* encodes a receptor for NGF but also binds NT-3 and NT-4/5 (7, 22, 26, 29, 31, 60). *TRK-B* encodes a receptor for BDNF but also binds NT-3 and NT-4/5 (7, 31, 60), and *TRK-C* encodes a receptor for NT-3 (33). The pattern of expression of each gene or protein in the developing nervous system of the mouse is specific, suggesting that each has a unique role (9, 30, 40, 58).

Recently, we found that most favorable neuroblastomas express high levels of *TRK-A* transcripts and that neuroblastoma cells expressing *TRK-A* can differentiate in response to NGF in culture (47, 48). In contrast, aggressive neuroblastomas, especially those with *N-myc* amplification, express

little or no detectable *TRK-A* mRNA, and many cell lines have a defective NGF receptor signaling pathway (2, 3, 39, 59). These results suggest that the biology of neuroblastomas is closely correlated with the developmental stages of the neurons from which the tumors originate, so neuroblastomas may be regulated in part by neurotrophic factors.

Here we report that a human neuroblastoma cell line, SMS-KCN, expresses both *TRK-B* and *BDNF* transcripts as well as *TRK-A* mRNA. BDNF induces immediate-early genes and phosphorylation of phospholipase C- γ 1 (PLC- γ 1), the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), and phosphatidylinositol-3 kinase (PI-3K) and appears to stimulate cell survival and neurite extension in an autocrine or paracrine manner. In contrast, NGF induces autophosphorylation of *TRK-A*, but the signaling pathway is deficient. Finally, truncated *TRK-B* appears to be preferentially expressed in more-differentiated tumors (ganglioneuromas and ganglioneuroblastomas), whereas full-length *TRK-B* is expressed in immature neuroblastomas with *N-myc* amplification.

MATERIALS AND METHODS

Cells and tissues. The derivations, descriptions, and culture conditions of cell lines were described previously (2). SMS-KCN cells, a gift from C. Patrick Reynolds (55), were maintained in the RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin (standard medium) at 5% CO₂-95% air at 37°C. Fresh tumor samples were obtained from 59 Japanese patients and 15 patients of the Pediatric Oncology Group in the United States. These 74 tumors were frozen on dry ice or in

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liquid nitrogen immediately after surgery (47, 48) and were stored at -80°C until use. Staging was done according to the method of Evans and colleagues (17). Excluding the 5 ganglioneuromas, 18 of the tumors were stage I, 11 were stage II, 12 were stage IV-S, 14 were stage III, and 14 were stage IV. Histologically, 5 were ganglioneuromas, 16 were ganglioneuroblastomas, and 53 were neuroblastomas. The histologies of the tumors were classified according to the guidelines proposed by the Japanese Pathological Society (54), which were based solely on the histological grade of differentiation of the neuroblastomas without involving age or other factors.

Neurotrophins, cDNA probes, and drugs. The following probes and reagents were generous gifts: recombinant human BDNF and NT-3 from AMGEN Pharmaceutical Co., Ltd.; mouse NGF and anti-NGF antibody from Eugene M. Johnson; the human *BDNF* probe from George D. Yancopoulos; the *N-myc* probe from J. Michael Bishop; probes for *TRK-A* and *TRK-B* from Luis Parada; the low-affinity nerve growth factor receptor (LNGFR) full-length cDNA probe from Moses Chao; and probes for human *NGFI-A*, *NGFI-B*, and *NGFI-C* from Jeffrey D. Milbrandt. The *TRK-A* probe was a 2.7-kb human cDNA fragment containing all of the coding region. The mouse *TRK-B* probe pFRK16 (32) recognizes mRNA transcripts both with and without the kinase domain (28, 43). The rat *TRK-C* probe pJDM836 contains a 557-bp PCR fragment corresponding to nucleotides 272 to 829 of the porcine *TRK-C* cDNA (33). K252a, an inhibitor of tyrosine phosphorylation (6, 52, 62), was purchased from Calbiochem Corp., La Jolla, Calif.

Northern (RNA) blot analysis. Total RNA was extracted from 0.2 to 1.0 g of cultured cells or frozen tumor tissue as described previously (10). We resolved 25 μg of each RNA on 1% agarose-formaldehyde gels and transferred the RNA by blotting to a nylon membrane (Hybond N+; Amersham, Arlington Heights, Ill.). Blots were hybridized, washed, and exposed to X-ray film as described previously (57). Northern analysis for expression of *TRK-B* and *BDNF* in the primary neuroblastomas was repeated three times.

Immunoprecipitations and immunoblots. Cells were grown in 15-cm-diameter dishes to $\sim 90\%$ confluency and incubated in serum-free medium for 1 h prior to NGF treatment. NGF-treated (100 ng/ml for 5 min) or untreated cells were then washed once with ice-cold Tris-buffered saline (TBS) (10 mM Tris [pH 8.0], 150 mM NaCl) and harvested by the addition of 300 μl of ice-cold lysis buffer (1% Nonidet P-40, 1 mM NaVO_4 , 0.1 mM NaMoO_4 , 1 mM phenylmethylsulfonyl fluoride, 3.3 μM pepstatin, 2 μM Bestatin, 10 μM leupeptin, 5.25 μg of aprotinin per ml, 0.02% NaN_3 in TBS) directly to the cell monolayer. BDNF- or NT-3-treated cells were exposed to 100 ng of BDNF or NT-3 per ml and treated in a manner similar to that for NGF-treated cells. Lysates were then immediately frozen and stored at -80°C . For the K252a studies, cells were incubated in serum-free medium with or without different concentrations of K252a for 6 h before the addition of 100 ng of BDNF per ml.

Prior to immunoprecipitation, lysates were thawed and incubated at 4°C for 15 min to ensure effective lysis, and cell debris was pelleted in a microcentrifuge (Eppendorf). Lysate supernatants were precleared by 1-h incubations with pre-immune rabbit serum and immobilized protein A bound to Sepharose beads (Repligen), in that order. The beads were spun down; anti-*TRK-A* antiserum (a generous gift from Luis Parada) or anti-*TRK-A* polyclonal immunoglobulin G (Santa Cruz Biotechnology, Inc.), both of which recognize both p140^{TRK-A} and p145^{TRK-B}, was added to the superna-

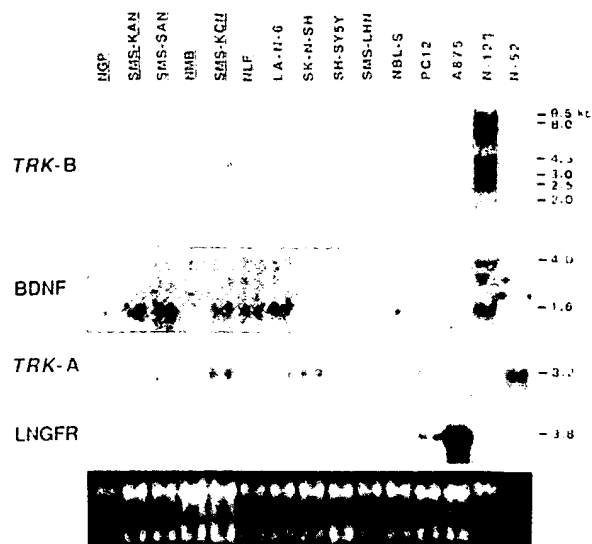


FIG. 1. Expression of *TRK-B*, *BDNF*, *TRK-A*, and *LNGFR* in 11 neuroblastoma cell lines, the PC12 rat pheochromocytoma cell line, and the A875 human melanoma cell line. For comparison of levels of *TRK-B* expression in primary tumors, total RNAs from a stage III primary neuroblastoma with *N-myc* amplification (N-123) and a stage I primary neuroblastoma (N-52) were loaded in the same gel. The underlines show the neuroblastoma cell lines or the primary tumor with *N-myc* amplification. The bottom panel is a photograph of the ethidium bromide-stained Northern blot gel.

tants; and incubations were performed at 4°C for ~ 16 h. Protein A beads were then added, and after 1 h at 4°C , the beads were pelleted and washed three times with lysis buffer and three times with TBS. The beads were then resuspended in $1\times$ Laemmli sample buffer and boiled for 5 min. The immunoprecipitates were then loaded onto 7.5% polyacrylamide gels for electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon) in cold 192 mM glycine–25 mM Tris base–20% (vol/vol) methanol for 45 min at 1 A. The blots were treated with 2% gelatin in TBS for ~ 45 min and were washed and then incubated with 1 μg of antiphosphotyrosine monoclonal antibody (UBI, Lake Placid, N.Y.) per ml in TBS–0.05% Tween 20 overnight. The antibody specifically bound to phosphotyrosine residues was visualized with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G secondary antibody (BMG) and a Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium detection system (Promega). These immunoprecipitates were then blotted, and the blot was probed with antiphosphotyrosine antibody (UBI).

RESULTS

Expression of neurotrophins and their receptors in neuroblastoma cell lines. Expression of *TRK-A*, *TRK-B*, *TRK-C*, *LNGFR*, *NGF*, and *BDNF* mRNAs in 11 human neuroblastoma cell lines, the PC12 rat pheochromocytoma cell line, and the A875 human melanoma cell line was studied by Northern blot analysis (Fig. 1). We also studied neurotrophic factor and receptor expression in a series of 74 neuroblastomas (see below). None of the cell lines except SMS-KCN (with *N-myc* amplification) expressed readily detectable levels of *TRK-B* transcripts. At least six different sizes of

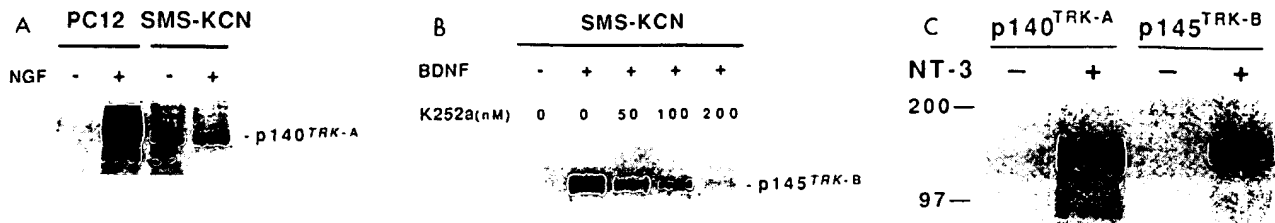


FIG. 2. Tyrosine phosphorylation of p140^{TRK-A} and p145^{TRK-B} in SMS-KCN human neuroblastoma cells. (A) Cells were either untreated (–) or treated with 100 ng of NGF per ml for 5 min at 37°C (+). The cell lysates were immunoprecipitated with anti-TRK-A antibody and then blotted with antiphosphotyrosine antibody (see Materials and Methods). (B) SMS-KCN cells were treated with 100 ng of BDNF per ml for 5 min at 37°C after incubation with the indicated concentrations of K252a for 6 h. The cell lysates were immunoprecipitated with anti-TRK-B antibody and blotted with antiphosphotyrosine antibody. (C) Cells were treated with 100 ng of NT-3 per ml for 5 min at 37°C. The cells were then immunoprecipitated with anti-TRK-A antibody or anti-TRK-B antibody and then blotted with antiphosphotyrosine antibody.

human *TRK-B* transcripts were detected in this cell line: 9.5, 8.0, 4.5, 3.0, 2.5, and 2.0 kb. BDNF, the primary ligand of p145^{TRK-B}, was expressed in four *N-myc*-amplified neuroblastomas (SMS-KAN, SMS-SAN, SMS-KCN, and NLF) and two nonamplified neuroblastomas (LA-N-6 and SMS-LHN). Two other neuroblastomas with *N-myc* amplification (NGP and NMB) also showed weak expression of *BDNF*. Neither *TRK-B* expression nor *BDNF* expression was detected in the SK-N-SH, SH-SY5Y, NBL-S, PC12, or A875 cell line.

Surprisingly, SMS-KCN cells expressed *TRK-A* at a level similar to that of PC12 cells, although *TRK-A* expression generally is down-regulated in *N-myc*-amplified neuroblastomas (47). NGF expression was observed only in the primary neuroblastoma N-123 (see below) but not in any cell lines tested (data not shown). *LNGFR* was expressed in all cell lines except five *N-myc*-amplified neuroblastoma cell lines (SMS-KAN, SMS-KCN, SMS-SAN, NMB, and NLF). None of the cell lines or primary neuroblastomas tested showed a detectable level of *TRK-C* expression, as measured by Northern analysis (data not shown).

BDNF induces phosphorylation of p145^{TRK-B} and expression of immediate-early genes in SMS-KCN cells. With SMS-KCN cells, NGF induced phosphorylation of p140^{TRK-A} (Fig. 2A) and BDNF induced phosphorylation of p145^{TRK-B} (Fig. 2B). The phosphorylation of p145^{TRK-B} induced by BDNF was inhibited by K252a in a dose-dependent manner, and inhibition was almost complete at 200 nM (Fig. 2B), which is similar to reports of inhibition of NGF-induced phosphorylation of p140^{TRK-A} (6, 62). The addition of NT-3 to SMS-KCN cells also induced receptor phosphorylation recognized by both anti-p140^{TRK-A} and anti-p145^{TRK-B} antibodies (Fig. 2C; see below). NGF induced none of the immediate-early genes in SMS-KCN cells (Fig. 3A), whereas BDNF, as well as NT-3, induced *c-FOS* and *NGFI-A* but not *NGFI-B* or *NGFI-C* (Fig. 3A and B).

These results contrast with the effect of NGF on PC12 cells, which is presumably mediated by p140^{TRK-A}. NGF induces phosphorylation of p140^{TRK-A} (26, 27, 29) and expression of immediate-early genes such as *c-FOS* (13, 18, 44), *NGFI-A/egr1/zif/268* (11, 45, 61), *NGFI-B/nur77* (21, 46), and *NGFI-C* (12). Primary cultures of neuroblastoma cells which express both functional p140^{TRK-A} and p75^{LNGFR} show a response to NGF stimulation similar to that of PC12 cells (48). However, no induction of immediate-early genes was seen in SMS-KCN cells (or other neuroblastoma cell lines tested) in response to NGF. Furthermore, BDNF and NT-3 induced *c-FOS* and *NGFI-A* but not *NGFI-B* or *NGFI-C*.

To perform these studies, we used two anti-p140^{TRK-A} antibodies from different sources, both of which recognized both p140^{TRK-A} and p145^{TRK-B} (data not shown). In contrast, the anti-p145^{TRK-B} antibody obtained from Santa Cruz Bio-

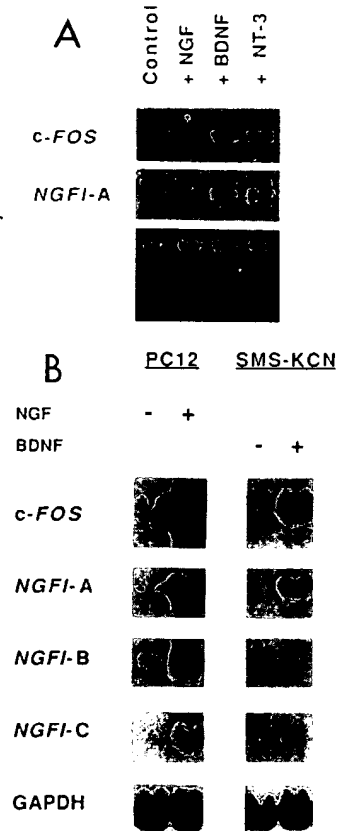


FIG. 3. Induction of expression of immediate-early genes in SMS-KCN cells. (A) Induction of *c-FOS* and *NGFI-A* in SMS-KCN cells in response to NGF, BDNF, or NT-3. Cells were treated with 100 ng of NGF, BDNF, or NT-3 per ml for 40 min at 37°C. The bottom panel is a photograph of the ethidium bromide-stained gel used for the Northern blotting. (B) Effect of NGF (in PC12 cells) or BDNF (in SMS-KCN cells) on induction of immediate-early genes *c-FOS*, *NGFI-A*, *NGFI-B*, and *NGFI-C*. Cells were treated with 100 ng of NGF or BDNF per ml for 40 min. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown for the normalization of expression of the other genes.

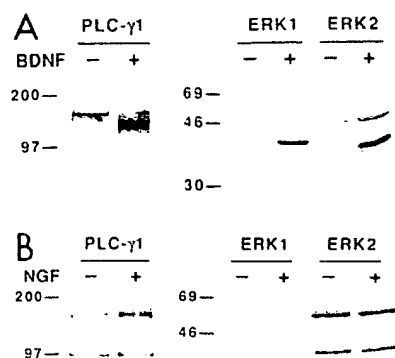


FIG. 4. Tyrosine phosphorylation of PLC- γ 1, ERK1, and ERK2 before (-) and after (+) the addition of BDNF (100 ng/ml) (A) or NGF (100 ng/ml) (B) to SMS-KCN cells for 5 min. Lysates were immunoprecipitated with specific antibody to each protein and blotted with antiphosphotyrosine antibody (see Materials and Methods).

technology almost exclusively recognized p145^{TRK-B} but was less sensitive than anti-p140^{TRK-A} antibodies in recognizing this receptor. A low level of p140^{TRK-A} phosphorylation was consistently observed in untreated SMS-KCN cell lysates after immunoprecipitation with anti-p140^{TRK-A} antibodies (Fig. 2A). Although some endogenous NGF- or NT-3-like molecule may stimulate p140^{TRK-A}, it is also possible that endogenously secreted BDNF stimulates p145^{TRK-B}, which is recognized by the cross-reacting anti-p140^{TRK-A} antibodies.

BDNF induces phosphorylation of PLC- γ 1, ERK1, ERK2, and PI-3K. NGF is reported to induce phosphorylation of PLC- γ 1 (64), ERK1 (8, 37), ERK2 (8), and PI-3K (53) in PC12 cells, and our results for all of these proteins were identical (data not shown). To determine whether BDNF induces a similar pattern of phosphorylation of these proteins in SMS-KCN cells, cell lysates were prepared after incubation of the cells with 100 ng of exogenous BDNF per ml for 1 or 5 min at 37°C, the lysates were immunoprecipitated with specific antibody, and the immunoprecipitates were Western blotted (immunoblotted) with antiphosphotyrosine antibody (Fig. 4). BDNF induced phosphorylation of PLC- γ 1 (150-kDa band), ERK1 (44-kDa band), and ERK2 (42-kDa band) after 5 min of incubation and induced a relatively weak level of phosphorylation of PI-3K (one 85-kDa band plus one 110-kDa band; data not shown) after 1 min of incubation (Fig. 4A). In contrast, NGF did not induce phosphorylation of either ERK1 or ERK2 in SMS-KCN cells (Fig. 4B), although induction of a minimum level of phosphorylation of PLC- γ 1 (Fig. 4B) and PI-3K (data not shown) was observed. The different effects of NGF and BDNF on protein phosphorylation in SMS-KCN cells are summarized in Table 1.

Effect of exogenous BDNF on growth and differentiation of SMS-KCN cells in vitro. The effect of exogenous neurotrophic factors (100 ng/ml) on the growth of SMS-KCN cells is shown in Fig. 5A. BDNF slightly increased the cell number during culture for longer than 7 days, while neither NGF nor NT-3 had a significant effect as determined by comparison with control cultures. Cell cycle analysis by flow cytometry showed that the treatment of SMS-KCN cells with 100 ng of exogenous BDNF per ml had no appreciable effect on the percentage of cells in different phases of the cell cycle (data

TABLE 1. Summary of protein phosphorylation in the BDNF/TRK-B signal transduction pathway in the SMS-KCN neuroblastoma cell line

Protein phosphorylated	Phosphorylation ^a by:			
	SMS-KCN		PC12	
	NGF	BDNF	NGF	BDNF
TRK-A	+	(-)	+	-
TRK-B	-	+	-	-
PLC- γ 1	±	+	+	ND
ERK1	-	+	+	ND
ERK2	-	+	+	ND
PI-3K	±	±	+	ND

^a +, strong induction; ±, weak induction; -, no induction; (-), negative result which cannot be confirmed absolutely because of cross-reactivity of TRK-A antibody with p145^{TRK-B}; ND, not determined.

not shown). This suggests that the increase in the cell number may be due to promotion of survival rather than stimulation of cell division.

We addressed the potential influence of serum concentration on cell growth or differentiation. SMS-KCN cells were grown at decreased serum concentrations in the culture medium, and as shown in Fig. 5B, cell growth was inhibited in a dose-dependent manner. However, the addition of exogenous BDNF increased the cell number at all serum concentrations. Even in the serum-free medium, there was half the starting number of cells after 8 days of culture with exogenous BDNF, though most cells were dead in its absence.

At every serum concentration, the presence of exogenous BDNF appeared to stimulate neurite outgrowth. In control medium with 10% serum, SMS-KCN cells spontaneously extended short neurites (Fig. 6A), which became straighter and longer as the cell density became higher. There appeared to be modest enhancement of neurite outgrowth in the presence of exogenous BDNF (Fig. 6B), but it was difficult to document. In medium with 0.5% serum, however, the cells survived without an increase in the cell number, and spontaneous neurite outgrowth was very limited (Fig. 6C). The addition of exogenous BDNF clearly increased the cell number and markedly enhanced neurite outgrowth (Fig. 6D).

Expression pattern of TRK-B and BDNF mRNAs in primary neuroblastomas. In contrast to results with neuroblastoma cell lines, expression of TRK-B was observed in 27 of 74 (36%) primary neuroblastomas (including five ganglioneuromas), but the expression pattern was complex (Fig. 7). By extrapolation from studies of the mouse and rat TRK-B mRNA species (32, 43), the ~9.5- and ~4.5-kb transcripts presumably encode the full-length product of human TRK-B, and other size transcripts encode the truncated form of the receptor without the tyrosine kinase domain. In each primary neuroblastoma expressing TRK-B, the intensities of both the ~9.5- and ~4.5-kb transcripts appeared to be equivalent. A clear ~9.5-kb transcript was found in 7 of 10 (70%) neuroblastomas with N-myc amplification and in only 2 of 64 (3%) tumors without amplification ($\chi^2 = 30.22$; $P < 0.001$) (Table 2). On the other hand, preferential expression of the putative truncated forms, especially of the ~8.0-kb transcript, was observed in 18 of 64 (28%) neuroblastomas without N-myc amplification. Interestingly, five of five (100%) ganglioneuromas showed preferential expression of the putative truncated form of TRK-B mRNA (Fig. 7; Table 2).

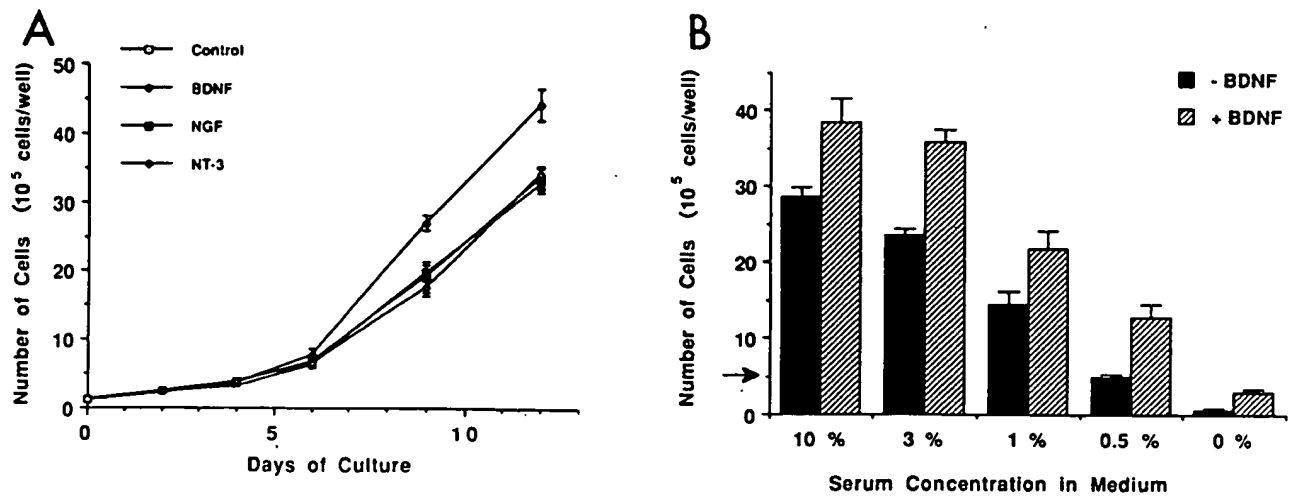


FIG. 5. (A) Effects of NGF, BDNF, and NT-3 on the growth of SMS-KCN cells. A total of 10^5 cells were seeded in a 24-well tissue culture plate (15-mm diameter; Falcon) and maintained in the complete medium, with changing every 2 to 3 days. The final concentration of each neurotrophic factor added was 100 ng/ml. The number of adherent cells was counted on day 8. Values are shown as means \pm standard deviations ($n = 4$). (B) Effects of serum concentrations on the growth of SMS-KCN cells in the presence or absence of 100 ng of BDNF per ml. A total of 5×10^5 cells per well (35-mm-diameter plate; Falcon) were plated on day 0 (arrow). Values for cell numbers on day 8 are shown as means \pm standard deviations ($n = 4$).

BDNF mRNA expression was detected in 50 of 74 (68%) primary neuroblastomas (Table 2). The frequencies were somewhat higher in advanced-stage (III or IV) neuroblastomas (22 of 28; 79%) and in mature ganglioneuromas (5 of 5;

100%) than in favorable-stage (I, II, or IV-S) neuroblastomas (23 of 41; 56%). Overall, 31% of the tumors had concordant expression of both *TRK-B* and *BDNF*. Seven of ten tumors with *N-myc* amplification had expression of both putative

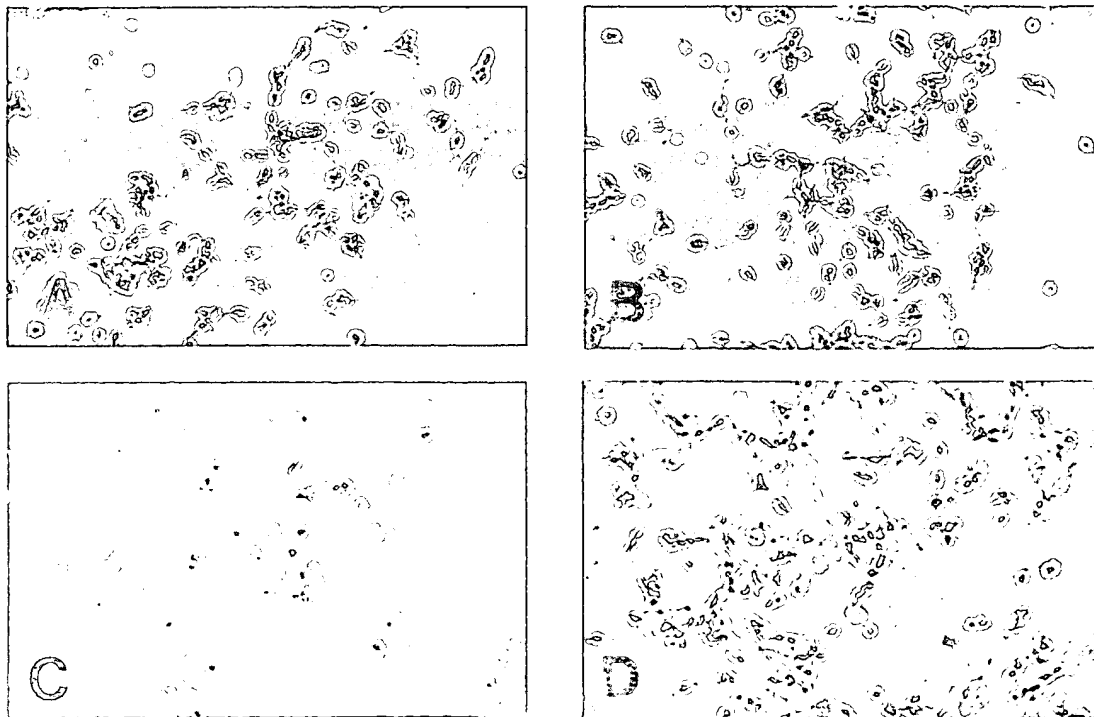


FIG. 6. Effects of BDNF on SMS-KCN cells cultured in the complete medium or in the 0.5% serum medium. (A and B) Cells were cultured in the 10% serum-RPMI 1640 medium for 5 days at 37°C at 5% CO₂-95% air without (A) or with (B) 100 ng of BDNF per ml. The initial cell concentration was 2×10^5 cells per ml. (C and D) Cells were cultured in the 0.5% serum medium alone (C) or with BDNF (D) for 8 days. The initial cell concentration was 5×10^5 cells per ml.

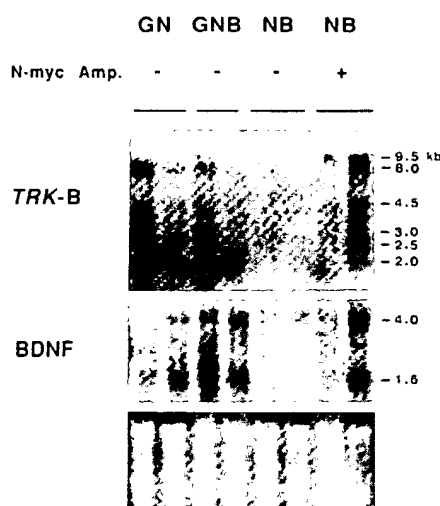


FIG. 7. Expression of *TRK-B* and *BDNF* in representative primary neuroblastomas. The bottom panel shows the ethidium bromide-stained gel used for the Northern blotting. GN, ganglioneuroma; GNB, ganglioneuroblastoma; NB, neuroblastoma; Amp., amplification.

full-length *TRK-B* and *BDNF*, similar to the expression in the SMS-KCN cell line. Of the remaining 64 tumors, 16 (25%) had coexpression of *BDNF* and *TRK-B*, and all but 2 of these expressed predominantly truncated forms.

DISCUSSION

The *BDNF/TRK-B* signaling pathway in the SMS-KCN neuroblastoma cell line is functional. In contrast to the *NGF/TRK-A* pathway, the *BDNF/TRK-B* signaling pathway in neuronal cells has been unclear because a suitable cell line has not been available. In the present article, we report that an *N-myc*-amplified neuroblastoma cell line, SMS-KCN, expresses readily detectable endogenous levels of both *TRK-B* and *BDNF* mRNAs. This cell line also expresses *TRK-A* but not *TRK-C*, *LNGFR*, or *NGF*.

In SMS-KCN cells, exogenous *BDNF* induced phospho-

rylation of p145^{TRK-B} and the downstream signaling pathway, including phosphorylation of PI-3K after 1 min of incubation, phosphorylation of PLC- γ 1, ERK1, and ERK2 after 5 min of incubation, and induction of the immediate-early genes *c-FOS* and *NGFI-A*. The signaling cascade is very similar to that of the *NGF/TRK-A* system in PC12 rat pheochromocytoma cells (8, 37, 53, 64), except for the absence of induction of *NGFI-B* and *NGFI-C*. Since *NGFI-B* is expressed in many primary neuroblastoma tissues (unpublished observations), the failure of this gene to be induced is probably not due to cell type or species differences.

BDNF seemed to promote cell survival and to induce neurite outgrowth of SMS-KCN cells. However, there are striking differences between the *BDNF/TRK-B* system in SMS-KCN cells and the *NGF/TRK-A* system in PC12 cells. *BDNF* may act on the SMS-KCN cells in an autocrine or paracrine manner, though it may not be saturated, while PC12 cells require exogenous *NGF* in order to differentiate. In addition, *BDNF* seems to induce neurite outgrowth and survival in SMS-KCN cells without substantial effects on cell growth, while *NGF* causes a decrease in the growth rate of PC12 cells. The presence of a *BDNF/TRK-B* autocrine loop in central and peripheral nervous systems has been suggested recently by some investigators on the basis of in situ hybridization studies (15, 58). Most primary neuroblastomas expressing both *BDNF* and *TRK-B* have *N-myc* amplification and overexpression, as shown in Table 2, and this is true for the SMS-KCN cell line as well. Thus, the potential autocrine stimulation of these cells through the *TRK-B/BDNF* pathway may be a consequence of *N-myc* overexpression.

SMS-KCN cells express *TRK-A* receptors as well, and *NGF*-induced phosphorylation of p140^{TRK-A} occurs. However, the downstream signaling cascade, including phosphorylation of ERK1 and ERK2 as well as induction of *c-FOS* and *NGFI-A*, was deficient. *NGF* also had no effect on morphological differentiation or tumor cell growth. Thus, as in some other neuroblastoma cell lines (2, 3, 39, 59), the *NGF/TRK-A* signal transduction pathway in SMS-KCN cells seems to be defective, although the similar signaling *BDNF/TRK-B* pathway is intact. PLC- γ 1 and PI-3K were weakly phosphorylated by the treatment of SMS-KCN cells with *NGF*. One of the explanations for this may be that there is a close association of these molecules with p140^{TRK-A} near the cell membrane (53). It is also possible that some signaling intermediate required for immediate-early gene induction is lacking or that there is some threshold of signal transduction beyond the membrane-associated substrates that is not exceeded by *NGF* interacting with its receptor.

The exogenously added NT-3 induced expression of immediate-early genes in SMS-KCN cells, probably through phosphorylation of the p145^{TRK-B} receptor (Fig. 2C). Although NT-3 also induced phosphorylation of p140^{TRK-A} (Fig. 2C), *NGF* was unable to induce immediate-early genes, so it is less likely that NT-3 would do so through this receptor. It is also possible that *TRK-C* was expressed below the level of detection or that another unknown receptor capable of binding NT-3 was responsible. However, the evidence that NT-3 could induce phosphorylation of p145^{TRK-B} and the fact that *BDNF* can induce immediate-early gene induction make this the most plausible mechanism.

Neuroblastomas expressing *TRK-A*, *TRK-B*, and/or *BDNF* may represent stages of the normal developmental neuronal lineage. The discovery of genes encoding neurotrophic fac-

TABLE 2. Expression of *TRK-B* and *BDNF* in primary neuroblastomas

No. of <i>N-myc</i> copies	Histology	Total	No. of cases ^a			
			With <i>TRK-B</i> mRNA expression		With <i>BDNF</i> mRNA expression	
			-	+	-	+
1	Ganglioneuroma	5	0	0	5	0
	Ganglioneuroblastoma	15	10	0	5	6
	Neuroblastoma	44	34	2	8	17
>1	Neuroblastoma	10	3	7	0	1
Total		74	47	9	18	24
						50

^a -, absent; +, present; full, tumors had ~9.5-kb signal intensity greater than or equal to the ~8.0-kb signal intensity; trunc., tumors had ~9.5-kb signal intensity less than the ~8.0-kb signal intensity.

tors and their receptors has provided considerable insight into the biology of neuroblastomas. In a previous study, we found that most primary neuroblastomas with favorable prognoses expressed a very high level of *TRK-A* mRNA, usually together with *LNGFR* mRNA. These tumor cells responded to NGF by terminally differentiating in vitro and died in the absence of NGF (47, 48). These observations suggested that tumor cells expressing functional NGF receptor may be susceptible to either programmed cell death, resulting in tumor regression, or to differentiation, leading to a benign ganglioneuroma, in vivo.

Our present results further show that many aggressive neuroblastomas, especially those with *N-myc* amplification, expressed both putative full-length *TRK-B* and *BDNF* mRNAs, although generally the expression of *TRK-A* mRNA was extremely low or absent. The almost mutually exclusive expression of *TRK-A* and *TRK-B* (with or without *BDNF*) suggests that neuroblastomas may be categorized into distinct subsets. Favorable tumors are composed mainly of cells expressing *TRK-A*, while aggressive tumors are composed of cells expressing both *TRK-B* and *BDNF*, often accompanied by *N-myc* amplification.

This hypothesis concerning the differential expression of *TRK-A* and *TRK-B* in neuroblastomas is consistent with the observations that neural crest-derived dorsal root ganglion cells normally require NGF and BDNF, as well as NT-3 and NT-4/5, to survive (5, 19, 23, 36). Furthermore, *TRK-A* and *TRK-B* transcripts are observed in distinct subsets of neurons in the dorsal root ganglia and sympathetic ganglia, as seen by in situ hybridization (9, 30, 40, 58). Finally, *BDNF* and/or *NT-3* transcripts are present in many neurons in both dorsal root ganglia and sympathetic ganglia, whereas *NGF* transcripts are not (16, 58). Thus, BDNF may stimulate responsive neurons by a local mechanism (15, 58), and it may be particularly important in providing trophic support to sensory neurons during the earliest phases of target innervation.

In the normal neuronal lineage, BDNF appears to support the survival of dopaminergic neurons (24). It is interesting that aggressive neuroblastomas with *N-myc* amplification often have dopaminergic characteristics (34, 49–51) and that those tumors often express both *TRK-B* and *BDNF*. In contrast, the putative truncated form of human *TRK-B* is preferentially expressed in more-differentiated ganglioneuroblastomas and ganglioneuromas. It is not clear whether the *TRK-B* expression of such tumors is derived from the differentiated neuroblastic tumor cells or from the Schwannian elements that are present in the differentiated tumors, because Schwann cells also are reported to express *TRK-B* and *BDNF* (1, 9). The roles of *NT-3* and *TRK-C* in neuroblastomas remain unclear. Remarkably, some primary cultures of neuroblastoma cells responded to NT-3 in a manner similar to that for NGF (unpublished observations). However, we have not detected expression of *TRK-C* in any primary neuroblastomas or cell lines (data not shown). This suggests that NT-3 may be acting through *TRK-A*, *TRK-B*, or another receptor.

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Minh-Tam DAULS

7) **Expression and function of TRK-B and BDNF in human neuroblastomas.**

Nakagawara A; Azar C G; Scavarda N J; Brodeur G M

Department of Pediatrics, Washington University School of Medicine, St.
Louis, Missouri 63110.

Molecular and cellular biology (UNITED STATES) Jan 1994, 14 (1)
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Art Unit 1642

8) ****Neurotrophins and Trk receptors in human pancreatic ductal adenocarcinoma: expression patterns and effects on in vitro invasive behavior.**

Miknyoczki S J; Lang D; Huang L; Klein-Szanto A J; Dionne C A; Ruggeri B
A

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9) **Differential cellular expression of neurotrophins in cortical tubers of the tuberous sclerosis complex.**

Kyin R; Hua Y; Baybis M; Scheithauer B; Kolson D; Uhlmann E; Gutmann D;
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10) **Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina.**

Ghazi-Nouri S M S; Assi A; Limb G A; Scott R A H; von Bussmann K;
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11) **Expression of NGF family and their receptors in gastric carcinoma: a cDNA microarray study.**

Du Jian-Jun; Dou Ke-Feng; Peng Shu-You; Qian Bing-Zhi; Xiao Hua-Sheng;
Liu Feng; Wang Wei-Zhong; Guan Wen-Xian; Gao Zhi-Qing; Liu Ying-Bin; Han
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Medical University, Xi'an, Shanxi Province, China.

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12) **Association of neurotrophin receptor expression and differentiation in human neuroblastoma.**

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13) **Expression of brain-derived neurotrophic factor and tyrosine kinase B receptor proteins in glioneuronal tumors from patients with intractable epilepsy: colocalization with N-methyl-D-aspartic acid receptor.**

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NEUROTROPHINS AND Trk RECEPTORS IN HUMAN PANCREATIC DUCTAL ADENOCARCINOMA: EXPRESSION PATTERNS AND EFFECTS ON *IN VITRO* INVASIVE BEHAVIOR

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The aggressive and highly metastatic behavior observed in pancreatic ductal adenocarcinoma (PDAC) may be due to autocrine and/or paracrine interactions (tumor/stromal) involving altered expression of peptide growth factors and their corresponding receptors. The neurotrophin (NT) growth factor family and their cognate receptors have been demonstrated to play a role in the invasiveness, chemotactic behavior and tumor cell survival of both neuronal and non-neuronal cancers. We hypothesized that aberrant expression of the NTs and/or the Trk receptors may contribute to the malignant phenotype of PDAC, specifically tumor cell invasiveness, through autocrine and/or paracrine interactions. In this study, we examined the expression of NTs, Trks and p75^{NGFR} by immunohistochemical and *in situ* hybridization analyses in both normal (*n* = 14) and neoplastic pancreas (*n* = 47) and PDAC-derived cell lines (*n* = 6). Further, we evaluated the effects of various NTs on the *in vitro* invasive and chemotactic behavior on 6 human PDAC-derived cell lines in a modified Boyden chamber assay. Brain-derived nerve growth factor (BDNF), NT-3, NT-4/5 and Trks A, B and C exhibited diffuse cytoplasmic and membranous immunostaining patterns in both the ducts and the acini of the exocrine pancreas and the islets of the endocrine pancreas of both normal and PDAC specimens. NT expression was primarily within the stromal compartment of the tumor, while Trk expression was weak or absent. We observed a 68%, 64% and 66% increase in the expression of Trks A, B and C, respectively, in the ductal elements of the PDAC samples examined compared with the normal adjacent tissue. Invasiveness of 4 of 6 PDAC cell lines was significantly inhibited (*p* < 0.05) when the cells were incubated with 100 ng/ml NT. However, when select cell lines were incubated with lower concentrations of NT-3 and BDNF (0, 1, 5, 25 and 50 ng/ml), invasiveness was significantly stimulated (*p* < 0.05) through the Matrigel matrix. Collectively, our data suggest the possibility that paracrine and/or autocrine NT-Trk interactions may influence the phenotype (possibly the invasive behavior) of PDAC. *Int. J. Cancer* 81:417–427, 1999.

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In the United States, pancreatic ductal adenocarcinoma (PDAC) represents the 4th leading cause of death in men and the 5th in women, with a 5-year survival rate of only 1–4% (Murr *et al.*, 1994; Noble and Goa, 1997). This high fatality rate is due to the “silent” progression of the disease until advanced stages, the lack of specific and sensitive markers for early detection and the largely refractory response to available surgical, chemotherapeutic and radiotherapeutic treatment modalities (Steele *et al.*, 1994). In approximately 45% of patients with pancreatic cancer the primary tumor has already metastasized by the time of diagnosis to distal sites including the liver, lungs, duodenum and peritoneum, while an additional 35–45% of patients present with unresectable or locally advanced disease. This aggressive and unchecked spread through lymphatic, hematogenous and notably perineural routes accounts for the rapid and fatal progression of the disease (Link *et al.*, 1997; Pour *et al.*, 1991).

A characteristic feature of PDAC is the production of a desmoplastic stromal compartment consisting primarily of fibroblasts and extracellular matrix, along with nerves, blood vessels and capillaries (Cormack, 1987; Lohr, 1996). This allows for the

possibility that these adjacent and infiltrating non-neoplastic tissues may influence tumor progression, vascularization and metastasis through the release of soluble mitogenic and chemotactic factors (Di Renzo *et al.*, 1995; Ebert *et al.*, 1995; Friess *et al.*, 1994; Lohr, 1996).

The neurotrophin (NT) family of growth factors—nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6) (Gotz *et al.*, 1994) and their cognate receptors [Trk A, B, C and the low-affinity nerve growth factor receptor (p75^{NGFR})] have been implicated in the paracrine growth regulation of a number of neuronal and non-neuronal tumor types (Miknyoczki *et al.*, 1996). Each NT binds to a specific Trk receptor: Trk A binds specifically to NGF; Trk B binds to both BDNF and NT-4/5; and Trk C binds to NT-3. All NTs bind with varying affinity to p75^{NGFR} (for reviews see Barbacid, 1995; Kaplan and Miller, 1997; Segal and Greenberg, 1996). While their principal biological function is to aid in the development and maintenance of the adult nervous system, NTs have also been shown to increase tumor invasiveness, enhance clonal growth and cause changes in cell morphology (Donovan *et al.*, 1994; Marchetti *et al.*, 1996; Oelmann *et al.*, 1995).

Specifically, in the development of malignant melanoma, NGF and NT-3 have been shown to aid in the metastasis of the malignant cells by increasing their production of extracellular matrix degrading enzymes. In addition, there is an increase in the density of p75^{NGFR} on the tumor surface as the melanoma becomes more invasive (Marchetti and McManaman, 1990; Marchetti *et al.*, 1993, 1996). In a similar manner, human prostatic carcinoma cells have been shown to be chemotactic (Djakiew *et al.*, 1993) and invasive (Geldof *et al.*, 1997) in response to NGF *in vitro*, and demonstrate a progressive loss of p75^{NGFR} with advancing tumor stage (Pflug *et al.*, 1995). Immunoreactivity for NGF has been demonstrated in human prostatic carcinomas (De Schryver-Kecsckemeti *et al.*, 1987) and tumor-derived cell lines (MacGrogan *et al.*, 1992), suggesting a possible mitogenic role or survival role for NGF in this cancer. In the growth of neuroblastoma (NB), the stimulation of Trk B by its ligand BDNF aids in the development and progression of the tumor by increasing invasiveness and tumor cell survival, inducing neurite extension, and cellular disaggregation (Matsumoto *et al.*, 1995). The failure to express Trk A receptors in NB also facilitates the early development of this tumor, by allowing neuroblasts to continually proliferate in an embryonic mode, acquiring genetic mutations or rearrangements that may eventually lead to tumor development (Brodeur, 1993).

Conversely, Trk C expression in medulloblastoma is associated with a favorable outcome (Lachyankar *et al.*, 1997). These collective data are illustrative of the fact that the expression patterns of specific NTs and their corresponding Trk receptors in specific cancers can have pronounced effects on the tumor pheno-

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type. To date, very little research has been performed on the role of NTs and Trk receptor expression in normal pancreatic tissue or in the development and progression of PDAC (Chiesa *et al.*, 1988; Kanaka-Gantenbein *et al.*, 1995; Koizumi *et al.*, 1998; Ohta *et al.*, 1997; Oikawa *et al.*, 1995; Scharfmann *et al.*, 1993; Shibayama and Koizumi, 1996). We have thus examined extensively the expression patterns and localization of expression of the NTs and their corresponding Trk and p75^{NGFR} receptors in a large series of normal pancreata, PDAC specimens and 6 human PDAC-derived cell lines by immunohistochemistry (IHC) and *in situ* hybridization. Further, we examined the *in vitro* invasive behavior of PDAC cell lines using a modified Boyden chamber assay demonstrating the functionality and biological relevance of NT-Trk interactions in this tumor type.

MATERIAL AND METHODS

Tissue samples

Human PDAC specimens and normal pancreata were obtained at autopsy or immediately following pancreatoduodenectomy, fixed in 10% buffered formalin and paraffin embedded. Details regarding the sources and acquisition of these clinical specimens have been described previously (Ruggeri *et al.*, 1997). All histologically confirmed ductal carcinomas were evaluated by the standard TMN staging system (Murr *et al.*, 1994) and from available pathology reports to determine tumor stage and grade. These data are summarized in Table I. Forty-seven PDACs and 19 normal pancreatic tissues were analyzed for expression of each Trk receptor subtype (Trk A, Trk B and Trk C) and the low-affinity NT receptor (p75^{NGFR}) and for expression of the NTs (NGF, BDNF, NT-3 and NT-4/5). Additional controls included IMR 90, a normal diploid lung fibroblast cell line (ATCC, Rockville, MD) that does not express either the NTs, the Trk receptors or p75^{NGFR}; PC12, a rat pheochromocytoma cell line (Greene and Tischler, 1976); and 3 stably transfected NIH3T3 fibroblast cell lines overexpressing rat Trks A, B and C (Camoratto *et al.*, 1997; Maroney *et al.*, 1997).

Tissue culture

Cell lines MiaPaca2, Panc 1, Capan 2 and HPAF (ATCC) and Colo 357 (Dr. Batra, Eppley Institute, Omaha, NE) were cultured in MEM with 10% FBS. BXPc3 and ASPC1 (ATCC) were cultured in RPMI with 20% FBS and the PC12 cell line (ATCC) was cultured in 5% FBS and 10% horse serum. The CFPAC cell line (ATCC) was cultured in DMEM with 10% FBS. The IMR 90 cell line (ATCC) was cultured in MEM with 10% FBS and 0.1 M non-essential amino acids. Transfected NIH3T3 fibroblasts expressing rat Trks A, B and C (Camoratto *et al.*, 1997; Maroney *et al.*, 1997) were cultured in DMEM with 10% FCS, G418 (400 µg/ml) and 2 mM glutamine. All cell lines were maintained at 37°C in 5% CO₂.

Source and characteristics of antibodies

Polyclonal Trks A, B and C (provided by Dr. D. Kaplan, McGill University, Montreal, Canada) were generated to peptides derived from intracellular domains of the specific Trk receptor subtype; the specificity and immunoreactivity of these antisera as determined by Western blotting have been described (Donovan *et al.*, 1994; Washiyama *et al.*, 1996). Additionally, the reactivity and specificity of these antibodies upon serial dilutions in IHC analyses were done as described previously (Donovan *et al.*, 1994; Hoehner *et al.*, 1995). Cep 21, a protein A-purified anti-pan Trk antibody made against the terminal 16 amino acids at the carboxy terminus of Trk, was also utilized. This antibody is Trk specific but does not discriminate between Trk receptor subtypes. The specificity of this antibody in IHC and Western blot analyses has been detailed previously (Camoratto *et al.*, 1997). These antibodies were titrated for use on tissue sections and optimized at a dilution of 1:100 with 5% normal goat serum. A monoclonal antibody (MAb) p75^{NGFR} (Boehringer Mannheim, Indianapolis, IN), was optimized at a 1:5

TABLE I - CLINICOPATHOLOGICAL CHARACTERISTICS AND IHC STAINING INTENSITY OF ARCHIVAL FORMALIN-FIXED PDAC¹

Case number	TMN stage ²	Grade ²	Pan Trk	Trk A	Trk B	Trk C
1	I (T ₁ N ₀ M ₀)	G2	++	++	+	+
2	III (T ₁ N ₀ M ₀)	G2	+/-	+	+/-	+/-
3	III (T ₃ N ₁ M ₀)	G2	+/-	+/-	+	+/-
4	I (T ₂ N ₀ M ₀)	G2	-	+/-	++	+/-
5	I (T ₂ N ₀ M ₀)	G3	+/-	+	+	+
6	III (T ₃ N ₁ M ₀)	NA ³	+/-	++	++	+
7	I (T ₁ N ₀ M ₀)	G3	+/-	+/-	-	+/-
8	III (T ₃ N ₁ M ₀)	G2	+/-	+	+/-	+
9	II (T ₃ N ₀ M ₀)	G2	+	+	+	+
10	I (T ₁ N ₀ M ₀)	G2	++	++	++	++
11	NA	G1	+/-	+	+/-	+
12	II (T ₃ N ₁ M ₀)	G1	+	+/-	+	+
13	IV (T ₁ N ₁ M ₁)	G2	+	+/-	+	+
14	III (T ₃ N ₁ M ₀)	G2	+	+	+	+
15	I (T ₂ N ₀ M ₀)	G3	+	+/-	+/-	+/-
16	IV (T ₂ N ₁ M ₁)	G3	+	++	+++	++
17	I (T ₁ N ₀ M ₀)	G3	+	++	+	+
18	III (T ₃ N ₁ M ₁)	G2	++	++	++	++
19	II (T ₃ N ₀ M ₀)	G2	+/-	++	+	++
20	IV (T ₃ N ₁ M ₁)	G2	++	++	++	++
21	II (T ₃ N ₁ M ₀)	G2	++	+++	++	++
22	III (T ₃ N ₁ M ₀)	G2	+	++	+	+
23	I (T ₂ N ₀ M ₀)	G2	+/-	-	-	-
24	III (T ₂ N ₁ M ₁)	G2	++	++	+	+/-
25	III (T ₃ N ₁ M ₀)	G3	+	+	++	+
26	III (T ₃ N ₁ M ₀)	G3	++	++	+	+
27	I (T ₁ N ₀ M ₀)	G2	++	++	+	+
28	I (T ₁ N ₀ M ₀)	G2	++	-	++	+
29	I (T ₁ N ₀ M ₀)	G2	++	+	+	++
30	III (T ₃ N ₁ M ₀)	G2	+	++	++	++
31	III (T ₃ N ₁ M ₀)	G1	+/-	-	+/-	+/-
32	III (T ₃ N ₁ M ₀)	G3	+/-	+/-	+	+/-
33	IV (T ₃ N ₀ M ₁)	G1	+/-	+/-	+/-	+/-
34	I (T ₂ N ₀ M ₀)	G1	+	+	+/-	+/-
35	IV (T ₂ N ₁ M ₁)	G2	+/-	+	+/-	++
36	II (T ₃ N ₀ M ₀)	G2	+	+	+/-	+/-
37	IV (T ₂ N ₁ M ₁)	G3	+	+	+	+
38	II (T ₃ N ₀ M ₀)	G2	++	+	+/-	+/-
39	I (T ₁ N ₀ M ₀)	G2	++	+/-	+	+/-
40	IV (T ₃ N ₁ M ₀)	G3	+	+	+	+
41	III (T ₃ N ₁ M ₀)	G2	-	+	+/-	+
42	III (T ₃ N ₁ M ₀)	G2	++	+	+/-	+
43	III (T ₃ N ₁ M ₀)	G3	-	+/-	+/-	-
44	I (T ₁ N ₀ M ₀)	G1	-	-	+/-	-
45	III (T ₃ N ₁ M ₀)	G1	+	+	+	+
46	III (T ₃ N ₀ M ₀)	G1	+/-	+/-	+/-	+
47	III (T ₃ N ₀ M ₀)	G2	+	+	+	+

¹PDAC specimens were analyzed using the ABC method (Vector) coupled with a 1.5% H₂O₂ block and microwave-based antigen retrieval (BioGenex). DAB in 0.03% hydrogen peroxide was used as the chromogen followed by counterstaining with hematoxylin. The level of immunostaining was based on staining intensity as defined in Material and Methods. ²Grade and staging information based on the TMN staging system. ³NA: information not available.

dilution. This MAb has been used in previously published reports at the same dilution (Bonetti *et al.*, 1997).

Polyclonal antisera immunospecific to NT-3, BDNF and NT-4/5 (provided by Dr. D. Kaplan) were titrated on tissue sections and optimized at a dilution of 1:500 with 0.3% Triton X-100. The characterization and immunoreactivity of these antisera as determined by Western blotting have been described (Washiyama *et al.*, 1996). Additionally, the reactivity and specificity of these antibodies upon serial dilutions in IHC analyses were done as described previously (Donovan *et al.*, 1994; Hoehner *et al.*, 1995). Similarly, for assessing NGF expression, an anti-NGF MAb (Promega, Madison, WI) was optimized at a dilution of 1:200 in 0.3% Triton X-100. The specificity of this MAb was tested by the manufacturer using an ELISA-based assay system.

IHC

Paraffin-embedded tissues were deparaffinized in xylene and rehydrated in a graded ethanol series. IHC was performed by the biotin-streptavidin-immunoperoxidase method (ABC Kit Elite; Vector, Burlingame, CA) using 3',3'-diaminobenzidine (DAB) in 0.03% hydrogen peroxide as a chromogenic agent. Endogenous peroxidases were blocked with 1.5% hydrogen peroxide, and non-specific staining was blocked with 5% normal goat serum or 5% normal horse serum. Microwave-based antigen retrieval protocols (BioGenex, San Ramon, CA) were employed on formalin-fixed tissue sections. A lead-based antigen retrieval solution was used for the determination of Trk expression, and a citrate-based antigen retrieval solution was used in the detection of NT expression. Phosphate-buffered saline (PBS), pH 7.6, was used in place of primary antibodies as an assay control to assess non-specific tissue reactivity. In addition, the specificity of each of the NT, Trk and p75^{NGFR} antibodies used was determined by preincubating each antibody with a 10-fold excess by weight of a competing peptide (Santa Cruz Biotechnology, Santa Cruz, CA). Human prostatic carcinoma specimens (Pflug *et al.*, 1995) served as positive controls for Trk receptors, p75^{NGFR} and the NTs. All immunostained tissues were counterstained with hematoxylin. Immunostaining patterns and intensity on tissue specimens were quantitated using a BioQuant (Ann Arbor, MI) Image Analysis system, and by histopathologic analysis. The level of immunostaining was based on the staining intensity and proportion of cells stained compared with the PBS control normal tissue and defined as described (Myers *et al.*, 1994; Ohta *et al.*, 1997). Briefly, if staining intensity was equal to the control normal adjacent pancreatic tissue the specimen was considered negative (-). Immunostaining greater than the control normal adjacent pancreatic tissue but limited to less than 25% of the cells in a tissue section was scored as weak (+); immunostaining intensity greater than the control and present in less than 50% of the cells was scored as moderate (++); and immunostaining intensity greater than the control and present in more than 50% of the cells was scored as strong (+++) (Myers *et al.*, 1994; Ohta *et al.*, 1997).

In situ hybridization and preparation of digoxigenin-labeled cRNA probes

Synthesis and preparation of digoxigenin-labeled riboprobes and oligoprobes. Plasmids containing the partial cDNA sequences for Trks A [human Trk A kinase domain base pairs (bp) 1420–2496], B (human Trk B kinase domain bp 2020–3350) and C (human Trk C kinase domain bp 1575–2800), and BDNF (entire human protein), NT-3 (mature human protein) and NT-4/5 (mature human protein) were generated in our laboratory and detailed previously (Camoratto *et al.*, 1997). Synthesis and purification of sense and antisense riboprobes were performed using the SP6/T7 DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's specifications. The yield of DIG-labeled probe was determined by comparison with a DIG-labeled control. Antisense and sense oligoriboprobes for β -NGF and p75^{NGFR} were synthesized (Bioserve Biotechnologies, Laurel, MD) from the following sequences corresponding to bp 641–670 of β -NGF (Ullrich *et al.*, 1983): antisense: 5' GUU GUU AAU GUU CAC CUC UCC CAA CAC CAU 3' NGF, sense: 5' UAC CAC AAC CCU CUC CAC UUG UAA UUG UUG 3'; and the sequence corresponding to bp 514–544 of p75^{NGFR} (Johnson *et al.*, 1986): antisense: 5' UCG UGU UCU CCU GCC AGG ACA AGC AGA ACA 3' p75^{NGFR} 5' ACA AGA CGA ACA GGA CCG UCC UCU UGU GCU 3' (Johnson *et al.*, 1986).

A DIG oligonucleotide 3'-end labeling kit (Boehringer Mannheim) was used to label the probes according to the manufacturer's specifications. An oligo d(T) probe (Novacastra, Newcastle upon Tyne, UK) was similarly prepared and used as a control to evaluate the quality of the mRNA in tissue specimens.

In situ hybridization. A modification of the protocols of Larsson *et al.* (1988) and Marchetti *et al.* (1995) was utilized. Paraffin-

embedded tissues (5 μ m) were deparaffinized in xylene, rehydrated in an ethanol series and washed in PBS. Tissue sections were incubated in 0.1 M glycine for 5 min, and 0.3% Triton X-100 for 15 min to reduce background. Slides were incubated for 30 min in proteinase K solution (10 μ g/ml), postfixed for 5 min in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride for 10 min. Tissue sections were incubated for 2 hr in a humidified chamber at 37°C with prehybridization solution containing 50% deionized formamide, 4 \times SSC, 1 \times Denhardt solution, 500 μ g/ml denatured salmon sperm, 250 μ g/ml yeast tRNA and 10% dextran sulfate. After prehybridization, slides were incubated overnight at 37°C in a humidified chamber with hybridization solution (prehybridization solution plus 100 pmol/ml probe). Posthybridization washes were as follows: 2 \times SSC for 15 min at 42°C (twice), 1 \times SSC for 15 min at 42°C (once), 0.5 \times SSC for 15 min at 42°C (twice) and 1 \times maleic acid buffer for 1 min at room temperature. Immunodetection of the signal was carried out by incubation with anti-digoxigenin alkaline phosphatase antibody (1:500) according to the manufacturer's protocol (Boehringer Mannheim).

In vitro invasion assay

The invasive potential of human PDAC-derived cell lines was analyzed using a modified Boyden chamber assay (Herrmann *et al.*, 1993). Transwell chambers (Fisher, Malvern, PA; 6.5 mm diameter polycarbonate membrane filter and 8 μ m pore size) were coated with 40 μ g/100 μ l growth factor reduced Matrigel (Fisher) and air dried under a laminar flow hood for 1 hr. Experimental cell lines (MiaPaca2, Panc 1, ASPC1, BXP3C, HPAF, CFPAC) and the control cell line (IMR 90) were stepped down to serum-free media (0.1% BSA, 5 ng/ml selenious acid and 5 μ g/ml transferrin) (Ebert *et al.*, 1995) over a period of 3 days (10% FBS 24 hr, 5% FBS 24 hr and serum-free media 24 hr) before starting the assays. Cells were resuspended in serum-free media, as above, and seeded in the upper chamber wells at a concentration of 5×10^4 cells/insert. Lower chamber wells contained specific NTs (Peprotech, Rocky Hill, NJ) at a concentration of 100 ng/ml (Djakiew *et al.*, 1993; Geldof *et al.*, 1997) and were incubated 24 hr at 37°C. Cell viability in response to the NTs tested was evaluated by Trypan blue staining. Non-invading cells were removed with a cotton tip applicator and invading cells were fixed and stained using the Diff-Quick staining kit (Allegiance Healthcare, McGraw Park, IL). The number of cells that penetrated through the Matrigel-coated filter and those that were found in the lower compartments were counted by light microscopy (counting 9 fields per filter and adjusting for the surface area of the filter) in triplicate assays. Chemotaxis experiments were carried out by setting up positive (0 ng/ml NT in the upper chamber and 100 ng/ml in the lower chamber) and negative (100 ng/ml NT in the upper chamber and 0 ng/ml in the lower chamber) concentration gradients of each NT as detailed (Djakiew *et al.*, 1993). Dose-dependent effects of NT-3 and BDNF on select cell lines were also performed in the same manner using 0, 1, 5, 25, 50 and 100 ng/ml NT. The specificity of the effect of NT-3 and BDNF on the *in vitro* behavior on select PDAC-derived cell lines was confirmed using neutralizing antibodies to NT-3 (0.30 μ g/ml NT-3 antibody in the presence of 5.0 ng/ml NT-3) and BDNF (30 μ g/ml in the presence of 5.0 ng/ml BDNF) (R&D Systems, Minneapolis, MN). Positive controls for PDAC cell invasive activity consisted of each cell line stimulated with 10% FBS. The IMR 90 cell line (normal human diploid lung fibroblast) was used as the negative control.

Statistical analysis

The statistical significance of the differences between serum-free conditions and incubation with NTs was tested by using one-way analysis of variance (ANOVA) and the Student-Newman-Keuls method (Sigma Stat for Windows; Jandel, San Rafael, CA). The values for the SEM represent the variance between triplicate replicates obtained from 3 independent experiments.

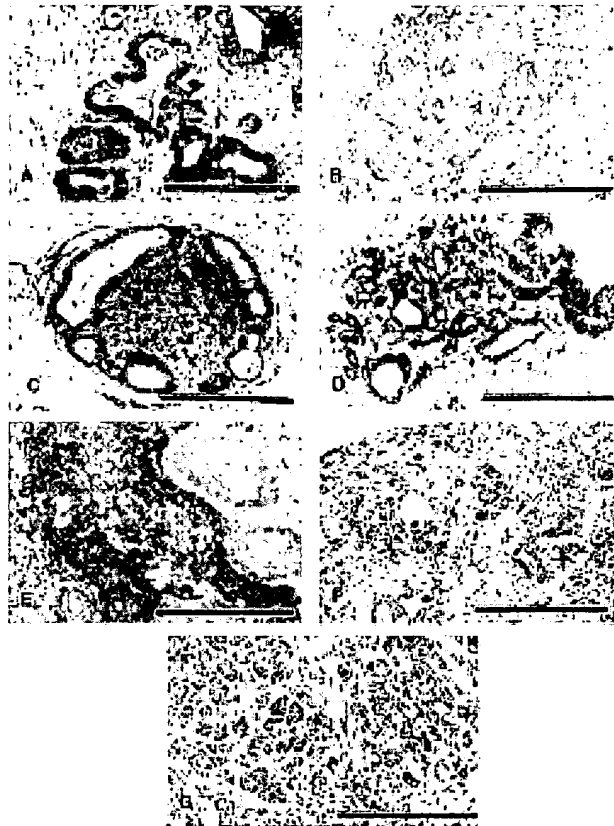


FIGURE 1—Immunolocalization of the NTs and Trk receptors in human PDAC. (a) Strong ductal immunostaining for the pan Trk antibody. (b) Moderate to strong ductal immunostaining for the Trk A antibody. (c) Moderate ductal immunostaining for the Trk B antibody, showing the presence of perineural invasion. (d) Moderate to strong ductal immunostaining for the Trk C antibody. (e) Weak ductal immunostaining for the NT-3 antibody, with moderate to strong immunostaining in the surrounding stroma. (f) Moderate ductal immunostaining for the NT-4/5 antibody. (g) Moderate acinar immunostaining for the BDNF antibody. Tissues were analyzed using the ABC method (Vector) with DAB in 0.03% hydrogen peroxide as the chromogen followed by counterstaining with hematoxylin. Bars: 70 μ m.

RESULTS

Results obtained from IHC using the specific antibodies titrated to obtain the optimum results revealed membranous and diffuse cytoplasmic immunostaining of pan Trk, Trks A, B and C, BDNF, NT-3 and NT-4/5 (Fig. 1). The pan Trk antibody was used to give an overview of total Trk immunoreactivity; individual Trk antibodies were used to characterize expression of individual Trk receptor subtypes in PDAC and normal adjacent pancreatic specimens. Immunostaining for the Trk receptors was present in the ductal elements of both the normal pancreata and PDAC specimens. However, compared with the normal adjacent tissue and normal pancreata, the PDAC specimens examined demonstrated increases of receptor immunostaining and cellular distribution of 61%, 68%, 64% and 66% for pan Trk and Trks A, B and C, respectively (Fig. 2). Trk immunoreactivity was not increased in adjoining normal pancreatic cells in tumor specimens. Moreover, areas of atrophic pancreas exhibited more intense immunostaining than normal pancreata parenchyma, but less than that observed in PDACs, suggesting the possibility that marked Trk receptor expression in pancreatic lesions is associated with the disease process and response to tissue damage. Immunostaining for p75^{NGFR} was weak to absent in both normal pancreata and PDAC tissues. However,

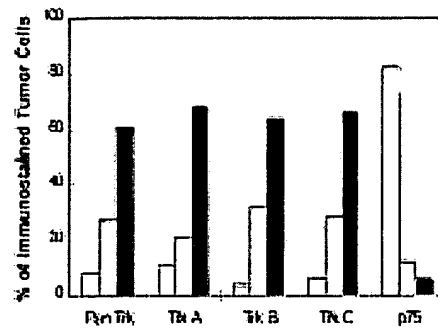


FIGURE 2—IHC staining intensity for Trk receptor isoforms and p75^{NGFR} in primary PDAC relative to normal adjacent tissues. White bars: negative immunostaining; gray bars: weak immunostaining; black bars: moderate to strong immunostaining. Immunostaining intensity and distribution are described in the Material and Methods and references.

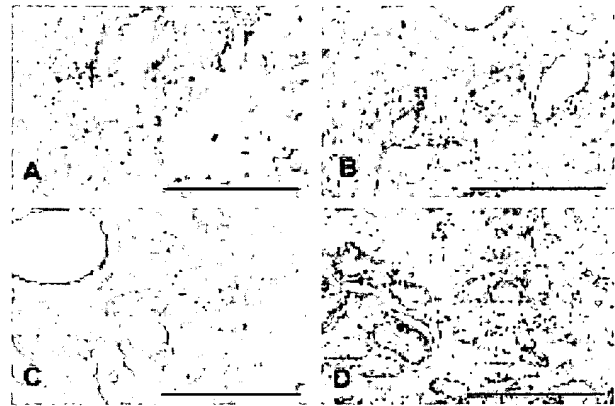


FIGURE 3—Controls and immunolocalization of NGF and p75^{NGFR}. (a) Pancreatic adenocarcinoma showing negative immunostaining for NGF. (b) Pancreatic adenocarcinoma showing negative immunostaining for p75^{NGFR}. (c) Prostate carcinoma showing moderate immunostaining for Trk A. (d) Prostate carcinoma showing moderate immunostaining for NT-3. Tissues were analyzed using the ABC method (Vector) with DAB in 0.03% hydrogen peroxide as the chromogen followed by counterstaining with hematoxylin. Bars: 40 μ m.

immunostaining was substantially less in PDAC tissues relative to normal pancreata (Fig. 3).

In contrast to Trk receptor expression patterns and immunostaining intensity, overall NT immunostaining intensity was not enhanced significantly between PDAC and normal adjacent pancreata, with the exception of BDNF, which exhibited an approximate 30% increase in immunostaining in PDAC specimens. The increase in BDNF immunostaining was not statistically significant. Immunostaining for NGF was absent in all normal and PDAC cases (Fig. 3). Immunostaining intensity for the various NTs was weak-moderate in pancreatic ducts and acini of normal and PDAC specimens, but markedly increased to varying degrees in the surrounding stromal elements of PDAC relative to normal pancreata. These data are suggestive that in contrast to normal pancreatic tissues, NTs are produced primarily by stromal elements of PDAC, and to a lesser extent the ductal carcinoma cells and normal acini within the tumor. Immunostaining of PDAC-derived cell lines revealed the absence of p75^{NGFR} staining, and moderate-strong immunostaining for Trks A, B and C in 4 of the 6 PDAC cell lines—Colo 357, MiaPaca2, Panc1 and HPAF. In general agreement with the primary PDAC data, immunostaining for BDNF, NT-3 and NT-4/5 was

TABLE II - IHC DATA: NT, Trk AND p75^{NGFR} EXPRESSION IN PDAC-DERIVED CELL LINES¹

Cell line	Pan Trk	Trk A	Trk B	Trk C	p75 ^{NGFR}	NGF	BDNF	NT-3	NT-4/5
BXPC3	+ ²	+/-	+ ³	+/-	-	-	+/++	+	+
Colo 357	+++	+++	+++	+++	-	-/+	+++	+++	+++
Capan 2	+/-	+	+	+/-	-	-	-/+	-/+	-/+
Panc 1	++	++	+	+	-	-	+/++	+/++	+/++
HPAF	++	++	+	+	-	-	++	+/++	+/++
MiaPaca2	++	+++	++	++	-	-	++/+++	++/+++	++
CFPAC	+++	ND ⁴	ND	ND	ND	ND	ND	ND	ND
ASPC1	+++	ND	ND	ND	ND	ND	ND	ND	ND

¹PDAC-derived cell lines were analyzed using the ABC method (Vector) as described for Table I. The level of immunostaining was based on staining intensity as defined in Material and Methods. -²Immunostaining seen on the periphery of the cells only. -³Immunostaining localized to the nucleus only. -⁴ND: not done.

weak to moderate intensity in the cell lines examined, while NGF immunostaining was negative (Table II).

In situ hybridization analyses utilizing digoxigenin-labeled riboprobes as well as oligomeric riboprobes corroborated our IHC findings on individual cases (n = 25) which were amenable for *in situ* hybridization analyses. Specifically, marked expression and localization of Trk A, B and C receptors in PDAC were observed primarily in the ductal elements of tumors, although some signal was likewise detected in scattered acini (Fig. 4). Expression of p75^{NGFR} as well as NGF was not detected in PDAC, possibly due to a poorly expressed or unstable message. The expression of BDNF and NT-3 was localized to both the ductal elements of the PDAC tumors and normal acini within these tumors (Fig. 4), while expression of NT-4/5 was primarily in acini. In addition, expression of BDNF and NT-3 was also pronounced in the tumor stroma of PDACs, while NT-4/5 was not observed in this area (data not shown). These data essentially corroborate our IHC findings using a series of anti-Trk and anti-NT antisera.

An unequivocal relationship between NT and Trk immunostaining patterns and intensity, *in situ* hybridization patterns and PDAC grade or TNM stage (Table I) could not be ascertained from our results. Moderate-strong immunostaining was observed in a majority of PDAC cases regardless of tumor grade or stage. Further, the disproportionate number of PDAC cases examined which were grade 2 (moderately well differentiated, n = 27) and TMN stages I and III (n = 33) complicate clear interpretation of NT and Trk expression in relation to grade and stage.

The invasive potential of human PDAC-derived cell lines through growth factor reduced Matrigel was determined using a modified Boyden chamber assay. At a concentration of 100 ng/ml of both BDNF and NT-3 there was a statistically significant ($p < 0.05$) inhibition of invasiveness for the BXPC3, ASPC1, Panc 1 and CFPAC cell lines compared with the serum-free control, while only BXPC3 and Panc 1 were significantly ($p < 0.05$) inhibited by 100 ng/ml of NGF (Fig. 5). At this concentration, no effect on invasiveness was observed in either the MiaPaca2 or HPAF cell lines. Table III summarizes the effects of each NT examined at 100 ng/ml on the invasive potential of the cell lines utilized in these studies. When the concentration gradient was reversed (100 ng/ml NT in the upper well), no statistical difference in cell migration was observed (data not shown). Thus, at 100 ng/ml, NTs are not a chemotactic attractant for human PDAC cells. Moreover, no adverse effects on cell viability were observed at the concentrations of NTs evaluated in these assays.

We then examined the dose-dependent effects of BDNF and NT-3 (0, 1, 5, 25, 50 and 100 ng/ml) on the invasiveness of select PDAC cell lines (MiaPaca2 and Panc 1 in response to BDNF, and ASPC1 and CFPAC in response to NT-3). At concentrations of 1, 5 and 25 ng/ml of BDNF and NT-3, Panc 1 and ASPC1, respectively, demonstrated a statistically significant ($p < 0.05$) increase in invasiveness compared with the serum-free control (Fig. 6). The CFPAC cell line showed a statistically significant increase in invasiveness ($p < 0.05$) in response to NT-3 at concentrations of 5 and 25 ng/ml (Fig. 6). No dose-dependent effects on invasiveness

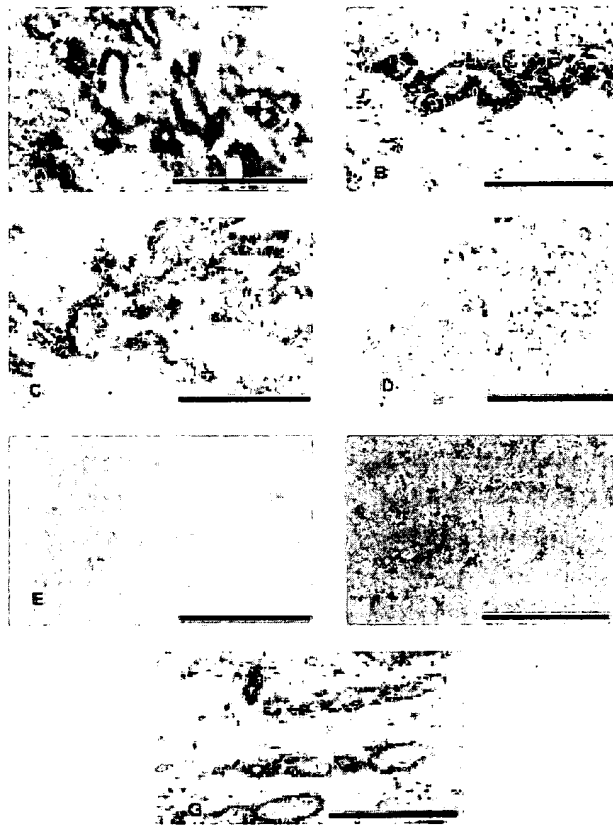


FIGURE 4 - Localization of NT, Trk receptors and p75^{NGFR} mRNA expression by *in situ* hybridization. (a) Localization of Trk A mRNA expression to the ducts of pancreatic adenocarcinoma. (b) Localization of Trk B mRNA expression to the ducts of pancreatic adenocarcinoma. (c) Localization of Trk C mRNA expression to the ducts of pancreatic adenocarcinoma. (d) Localization of NT-3 mRNA expression to the acinar cells of pancreatic adenocarcinoma. (e, f) Negative expression of p75^{NGFR} (e) and NGF (f) in pancreatic adenocarcinoma. (g) Oligo(dT) control used to test the quality of the mRNA present. Digoxigenin-labeled RNA probes were detected using NBT/X phosphate as chromogen. Bars: 65 μ m.

were observed with the MiaPaca2 cell line in response to BDNF, which is in agreement with the lack of response seen at higher concentrations of NT as summarized in Table III. From these data, we chose to determine the specificity of effects of BDNF and NT-3 (5 ng/ml) on the invasiveness of PDAC cells when incubated with neutralizing antibodies against BDNF and NT-3. No statistically significant differences were observed between tumor cells incubated with serum-free media and tumor cells incubated with NT

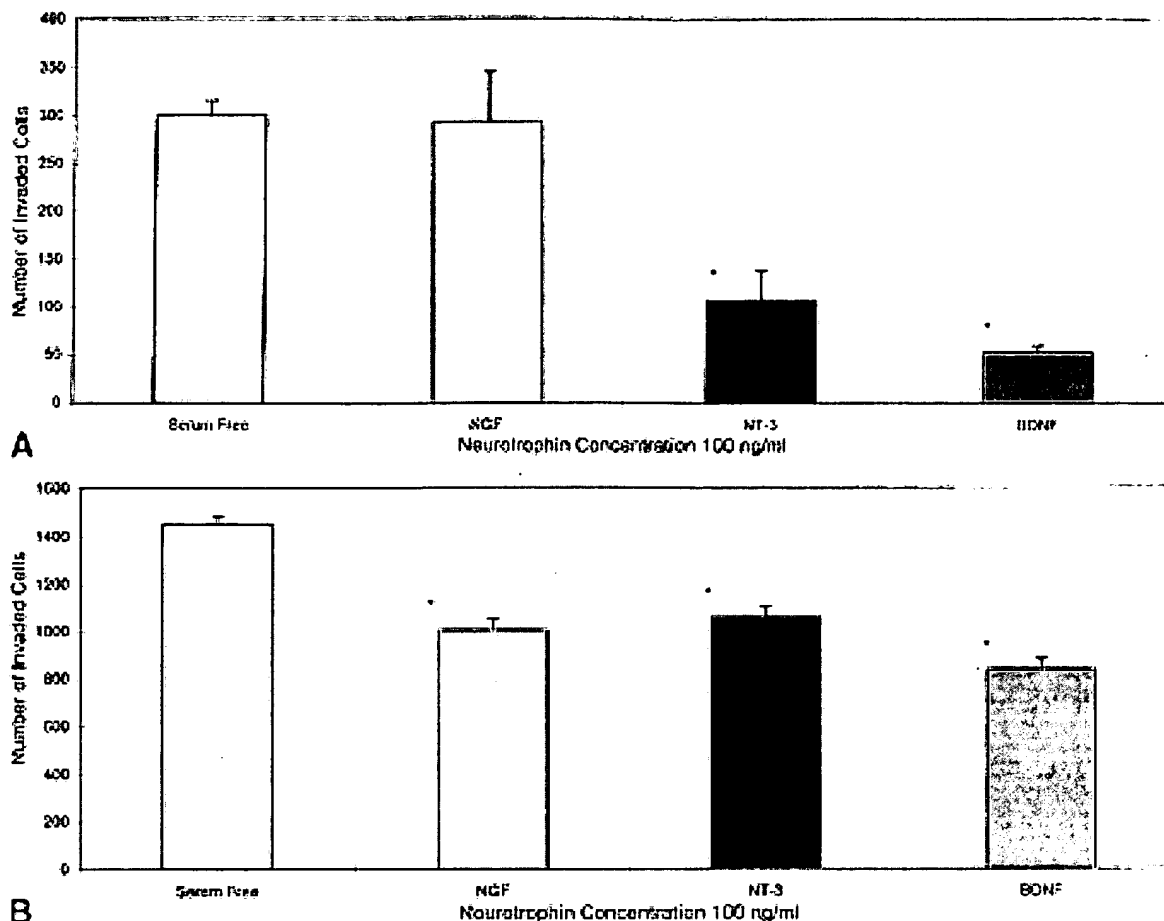


FIGURE 5 – Effects of NTs (100 ng/ml) on the *in vitro* invasiveness of human PDAC-derived cell lines: CFPAC (a), Panc 1 (b), ASPC1 (c) and BXP3 (d) using a modified Boyden chamber assay with growth factor reduced Matrigel; 5×10^4 cells/insert were incubated with 100 ng/ml of specific NT for 24 hr at 37°C. Non-invading cells were removed and invading cells were fixed and stained using the Diff-Quick staining kit. The number of invading cells that penetrated the Matrigel-coated filter and those that were found in the lower compartment were counted by light microscopy (see Material and Methods). * $p < 0.05$ using one-way ANOVA and Student-Newman-Keuls method.

with neutralizing anti-BDNF or anti-NT-3 alone, and in the presence of exogenous BDNF and NT-3, respectively (Fig 6). These data indicate that the effects observed are NT-Trk specific.

DISCUSSION

In this report, we have examined the expression patterns and localization of an additional novel growth factor-receptor axis potentially involved in the development of PDAC, *i.e.*, involving the NTs and their corresponding Trk receptor subtypes in the tumor-stroma interactions characteristic of PDAC. We have demonstrated the expression and localization of expression of BDNF, NT-3, NT-4/5 and the Trk receptors (Trks A, B and C) in normal and neoplastic pancreas as well as human PDAC-derived cell lines. IHC analyses revealed the presence of marked membranous and diffuse cytoplasmic staining for the Trk receptors relative to normal adjacent pancreata primarily in the ducts of PDAC. Immunostaining for various NTs was present in the surrounding tumor stroma. However, NT immunostaining was also present in ducts as well as acinar elements. Moreover, the antibodies employed in these IHC studies recognize epitopes at or near the carboxy termini of the Trk receptors making it likely that the detected proteins have full-length functional tyrosine kinase domains. This was corroborated by the detection by immunoprecipitation and Western blotting of autophosphorylated Trk receptor expression in NT-stimulated

PDAC cell lines using these antisera (data not shown). The pattern of immunostaining observed in primary PDAC specimens is not unique for the Trk receptor family, but has been described for other receptor tyrosine kinases as well. For example, expression of erb-B-2 and erb-B-3 receptors revealed both cytoplasmic and membranous immunostaining in prostatic intraepithelial neoplasia and prostatic adenocarcinoma (Myers *et al.*, 1994). Cytoplasmic immunostaining for the *c-met* (Ebert *et al.*, 1994a) and acidic (a) and basic (b) fibroblast growth factor (FGF) receptors (Kobrin *et al.*, 1993) has also been demonstrated in PDAC specimens.

In human normal non-neuronal tissues, IHC analysis reveals that Trks A and C are expressed at the intercalated/intralobular ducts of the pancreas and Trk B by the α -cells of the endocrine pancreas (Shibayama and Koizumi, 1996). Our results are in agreement with these results, with the exception of Trk B, which we found to be expressed in the ducts. Another study examined the expression of Trk A only in a series ($n = 11$) of PDAC specimens by IHC. These data showed that only 27% of the specimens were positive for Trk A expression (Koizumi *et al.*, 1998). The discrepancy between these results and ours may be due to the fact that only a small sample size was used and the only method for detection of expression was by IHC. In this study, we demonstrated the expression of all isoforms of the Trk receptors using 2 different experimental techniques (IHC and *in situ* hybridization) and

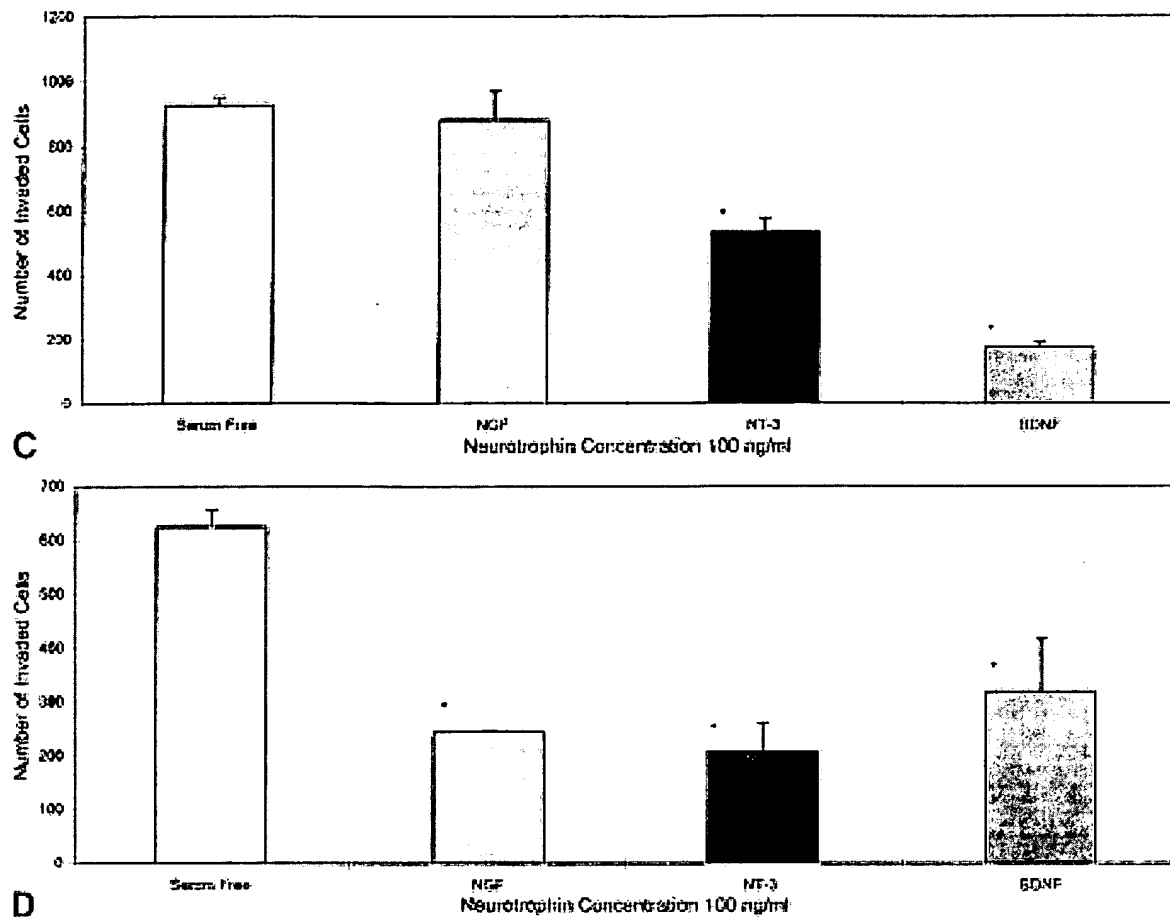


FIGURE 5

TABLE III—SUMMARY OF EFFECTS OF NT INCUBATION ON THE *IN VITRO* INVASIVE POTENTIAL OF HUMAN PDAC-DERIVED CELL LINES¹

Cell line	NGF (100 ng/ml)	NT-3 (100 ng/ml)	BDNF (100 ng/ml)
ASPC1	NE ² —invasiveness	↓ Invasiveness ³	↓ Invasiveness ³
BXPc3	↓ Invasiveness ³	↓ Invasiveness ³	↓ Invasiveness ³
CFPAC	↓ Invasiveness	↓ Invasiveness ³	↓ Invasiveness ³
HPAF	NE—invasiveness	NE—invasiveness	NE—invasiveness
MiaPaca2	NE—invasiveness	NE—invasiveness	NE—invasiveness
Panc 1	↓ Invasiveness ³	↓ Invasiveness ³	↓ Invasiveness ³

¹Positive controls used were PDAC cells stimulated with 10% FBS. —NE: no effect. —³Significant decrease compared with serum-free controls using one-way ANOVA and Student-Newman-Keuls method.

utilizing a larger sample size: n = 47 (PDAC specimens) and n = 14 (normal pancreata).

Our hypothesis for possible paracrine (tumor-stromal) influences of NT-Trk interactions aiding in the development of PDAC is further supported by the findings of Ohta *et al.* (1997), who examined the effects of NT-3 on PC12 cell lines. Their results suggest that PDAC cells synthesize and secrete NT-3 into the surrounding stroma enhancing neurite outgrowth of PC12 cells. Further, NT-3 immunostaining was localized to the ductal carcinoma cells, and a correlation was made between advanced tumor stage (stages III and IV) and NT-3 expression. Such correlations of NT or Trk receptor expression and tumor stage or grade from our

data were not possible, due to the fact that the majority of PDAC specimens examined were from TMN stages I and II (n = 33).

Aberrant expression of growth factors and their receptors is well known to contribute to the malignant phenotype of many tumors via autocrine and/or paracrine pathways, including PDAC (Ebert *et al.*, 1994b; Friess *et al.*, 1994). A histologic feature of PDAC is its prominent stromal compartment, which allows for the possibility that adjacent non-neoplastic tissues might influence PDAC growth and development through the release of soluble factors via paracrine interactions. This interaction is exemplified by the production of hepatocyte growth factor (HGF) by surrounding mesenchymal cells and the expression of the *c-met*-encoded HGF receptor on adjacent PDAC cells (Di Renzo *et al.*, 1995). Similarly, both paracrine and autocrine influences can be attributed to aFGF and bFGF, which are produced by fibroblasts and endothelial cells in the surrounding tumor stroma as well as by PDAC cells, with the corresponding high-affinity FGF receptor subtypes 1 and 2 expressed on the tumor cell surface (Leung *et al.*, 1994). Examples of autocrine growth pathways in PDAC are those involving the epidermal growth factor family (EGF) of mitogenic peptides [EGF, transforming growth factor α (TGF α), cripto and amphiregulin] and the overexpression on PDAC cells of the EGF receptor through which they exert their mitogenic and trophic effects (Davies *et al.*, 1993; Ebert *et al.*, 1994b; Friess *et al.*, 1994; Sandgren *et al.*, 1990). The concurrent presence of the EGF receptor and TGF α in PDAC has been associated with increased tumor invasiveness and shorter survival time following surgical resection (Friess *et al.*, 1994). Similar findings have been reported for aFGF, bFGF and

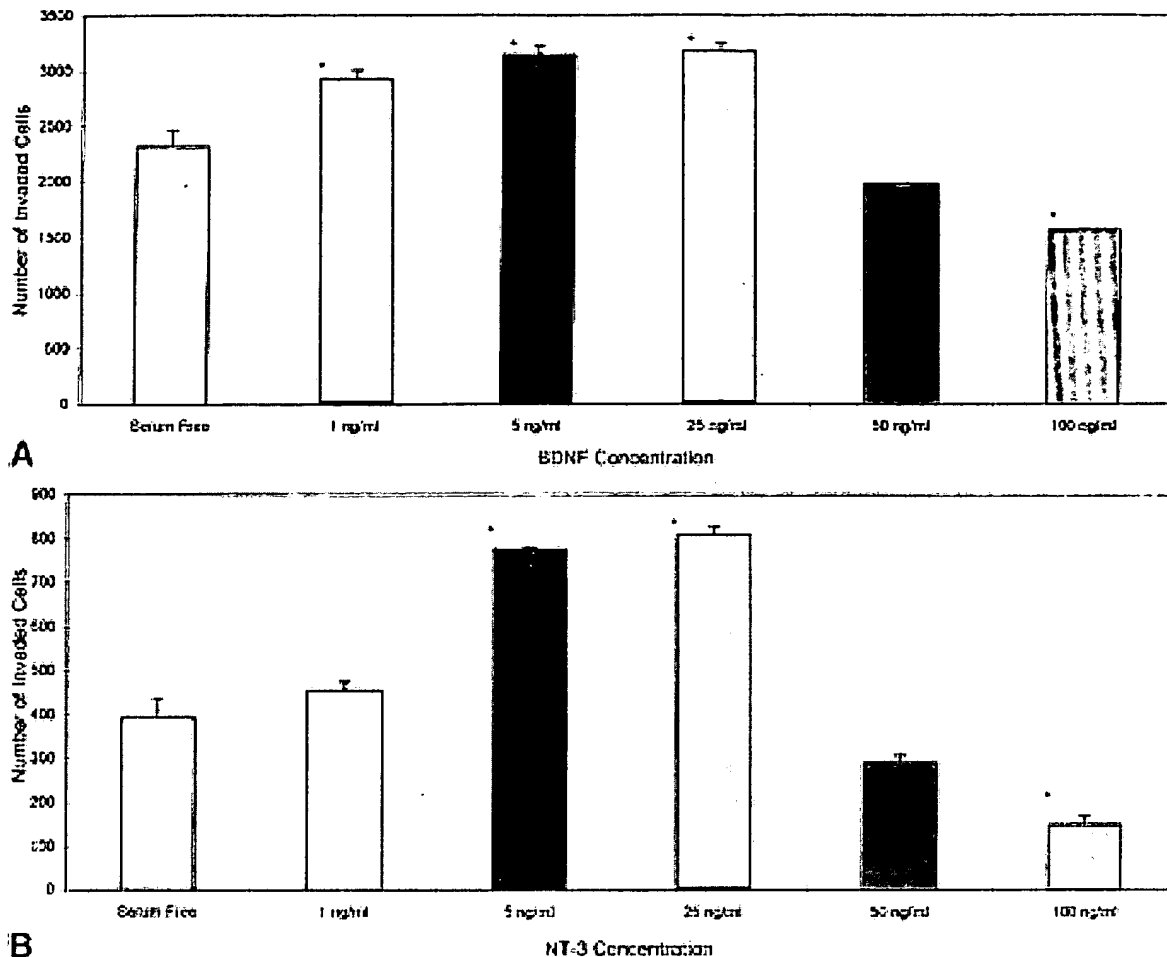


FIGURE 6 – Dose-dependent effects of BDNF on the Panc 1 cell line (a), the dose-dependent effects of NT-3 on the CFPAC (b) and ASPC1 (c) cell lines and the effects of BDNF and NT-3 incubated with their respective neutralizing antibodies (d) using a modified Boyden chamber assay with growth factor reduced Matrigel; 5×10^4 cells/insert were incubated with 100 ng/ml of specific NT for 24 hr at 37°C. Non-invading cells were removed and invading cells were fixed and stained using the Diff-Quick staining kit. The number of invading cells that penetrated the Matrigel-coated filter and those that were found in the lower compartment were counted by light microscopy (see Material and Methods). * $p < 0.05$ using one-way ANOVA and Student-Newman-Keuls method.

amphiregulin and/or cripto in PDAC (Yamanaka *et al.*, 1993; Yokoyama *et al.*, 1995).

Altered Trk expression may be an additional receptor tyrosine kinase that is aberrantly expressed in PDAC. We examined the effects of NT-Trk interactions on the invasive potential of PDAC using concentrations of NTs similar to what has been examined in prostate carcinoma, malignant melanoma, human small cell lung carcinoma and human breast carcinoma in *in vitro* studies (Descamps *et al.*, 1998; Djakiew *et al.*, 1991, 1993; Herrmann *et al.*, 1993; Marchetti *et al.*, 1996; Marchetti and Nicholson, 1997; Missale *et al.*, 1998). Using a modified Boyden chamber assay, we have demonstrated a significant decrease ($p < 0.05$) in the *in vitro* invasiveness of ASPC1, CFPAC, BXP3 and Panc 1 cell lines in response to 100 ng/ml BDNF and NT-3 as well as a significant decrease ($p < 0.05$) in invasiveness of BXP3 and Panc 1 cells in response to 100 ng/ml NGF. However, at lower concentrations (1, 5 and 25 ng/ml) of NTs (BDNF and NT-3), we observed a reproducible significant increase ($p < 0.05$) in invasiveness with some of the same cell lines (Panc 1, ASPC1 and CFPAC), the specificity of which could be blocked upon prior incubation with anti-BDNF and anti-NT-3 neutralizing antisera. The reasons for the dose-dependent

effects of NTs on PDAC *in vitro* invasiveness are not clear. Similar *in vitro* dose-dependent effects have been seen with malignant melanoma cell lines (Herrmann *et al.*, 1993; Marchetti *et al.*, 1993). No correlation could be made regarding the *in vitro* invasive behavior of the cell lines, Trk expression, or differentiation status when grown *in vivo*. This observation may be a result of the creation of a negative feedback system in which the cells' response to the NTs is altered when exposed to high concentration of exogenous NT (Herrmann *et al.*, 1993). Such an effect has been suggested for 70W and MeWo melanoma cells in response to NGF, and has been demonstrated for the effect of heparin binding growth factor on the proliferation of HepG2 cells where low concentrations are stimulatory and high concentrations are inhibitory (Battegay *et al.*, 1990; Herrmann *et al.*, 1993). Alternatively, the kinetics of activation of the signal transduction pathways utilized upon NT stimulation may result in expression of different genes (immediate early genes vs. delayed response genes), thereby giving the different responses observed in our study (Greene and Kaplan, 1995; Segal and Greenberg, 1996).

Our data extend the body of evidence that NT-Trk receptor interactions may have effects on the development and progression

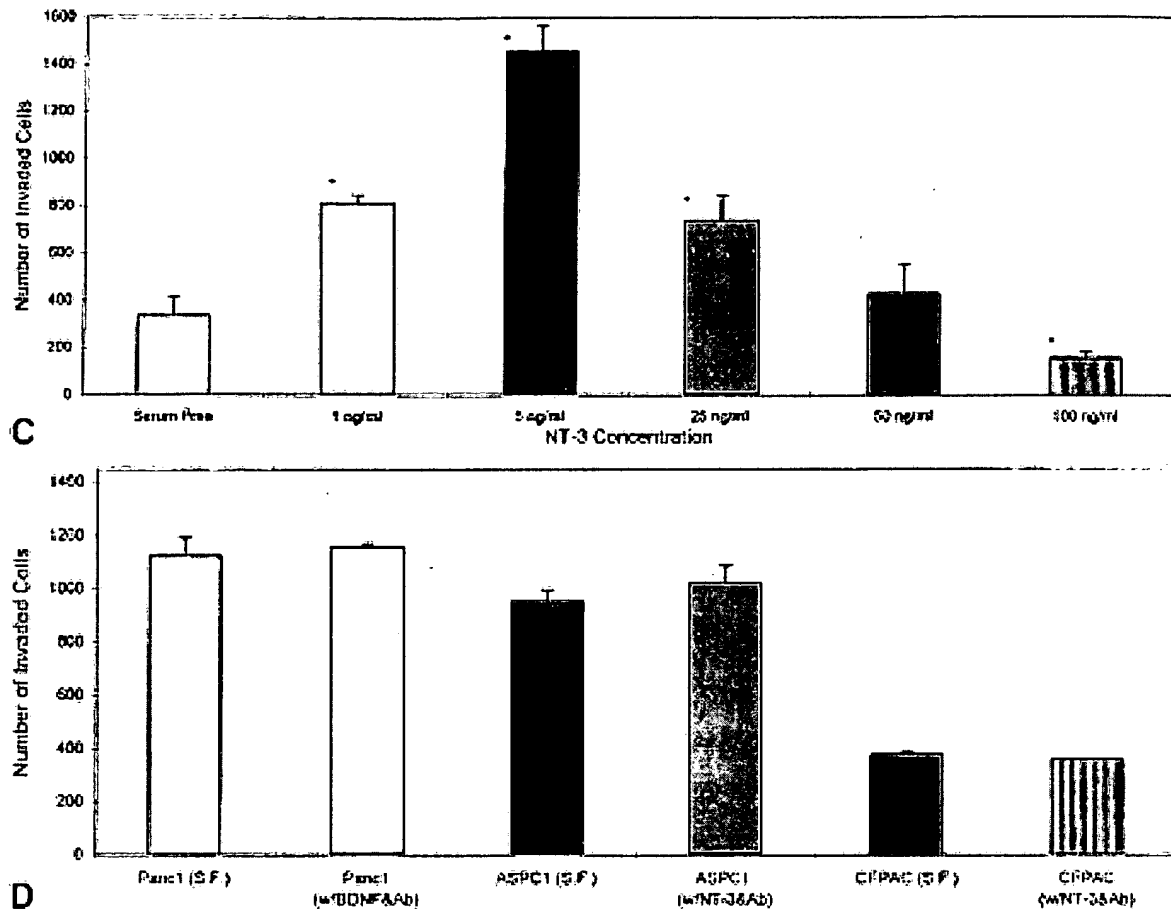


FIGURE 6

of many non-neuronal tumors. For example, human prostate carcinoma cell lines have been found to be chemotactic in response to human prostate secretory proteins that contained an NGF-like protein (De Schryver-Kecsckemeti *et al.*, 1987; Djakiew *et al.*, 1991; MacGrogan *et al.*, 1992). Similarly, NGF and NT-3 have been shown to upregulate heparanase production in brain metastatic melanoma cells. This upregulation of specific NTs has been shown to provide the melanoma cells with an increased ability to invade and metastasize through the extracellular matrix (Marchetti *et al.*, 1993, 1996). p75^{NGFR} has also been implicated as having a role in the mediation of perineural spread of desmoplastic melanoma, whereby injured axons secrete NGF or BDNF and cause the tumor cells to migrate toward the nerve (Iwamoto *et al.*, 1996). While no functional activity has yet to be established, altered expression of NTs and Trks has also been identified in Wilms's tumor (Donovan *et al.*, 1994), thyroid and colon carcinomas (Lambelle *et al.*, 1992) and cancers of the bile duct, gallbladder and rectum (Ohta *et al.*, 1997).

In conclusion, aberrant expression of the major isoforms of the Trk receptor (Trks A, B and C) has been demonstrated in PDAC specimens. Further, the dose-dependent effects of specific NTs on the *in vitro* invasiveness of select PDAC-derived cell lines suggest that physiological concentrations of NTs can influence the biological behavior (invasiveness) of this tumor type. The feasibility and antitumor efficacy of using potent and selective Trk receptor tyrosine kinase inhibitors have been demonstrated as a therapeutic strategy against specific cancers (Camoratto *et al.*, 1997; Dionne *et al.*, 1998). The results from these studies and our own findings may lead to possible treatments in the management of PDAC by using selective Trk-specific inhibitors to uncouple NT-Trk interactions *in vivo*.

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Art Unit 1642

7) **Expression and function of TRK-B and BDNF in human neuroblastomas.**
Nakagawara A; Azar C G; Scavarda N J; Brodeur G M
Department of Pediatrics, Washington University School of Medicine, St.
Louis, Missouri 63110.

Molecular and cellular biology (UNITED STATES) Jan 1994 , 14 (1)
p759-67, ISSN 0270-7306--Print Journal Code: 8109087
Contract/Grant No.: CA-39771; CA; NCI; CA-49712; CA; NCI

8) ****Neurotrophins and Trk receptors in human pancreatic ductal adenocarcinoma: expression patterns and effects on in vitro invasive behavior.**

Miknyoczki S J; Lang D; Huang L; Klein-Szanto A J; Dionne C A; Ruggeri B
A

Department of Pathology, MCP-Hahnemann University, Philadelphia, PA, USA.
International journal of cancer. Journal international du cancer (UNITED
STATES) May 5 1999 , 81 (3) p417-27, ISSN 0020-7136--Print
Journal Code: 0042124

9) **Differential cellular expression of neurotrophins in cortical tubers of the tuberous sclerosis complex.**

Kyin R; Hua Y; Baybis M; Scheithauer B; Kolson D; Uhlmann E; Gutmann D;
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American journal of pathology (United States) Oct 2001 , 159 (4)
p1541-54, ISSN 0002-9440--Print Journal Code: 0370502
Contract/Grant No.: K08-01658; PHS

10) **Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina.**

Ghazi-Nouri S M S; Assi A; Limb G A; Scott R A H; von Bussmann K;
Humphrey I; Luthert P J; Charteris D G

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British journal of ophthalmology (England) Apr 2003 , 87 (4)
p488-92, ISSN 0007-1161--Print Journal Code: 0421041

11) **Expression of NGF family and their receptors in gastric carcinoma: a cDNA microarray study.**

Du Jian-Jun; Dou Ke-Feng; Peng Shu-You; Qian Bing-Zhi; Xiao Hua-Sheng;
Liu Feng; Wang Wei-Zhong; Guan Wen-Xian; Gao Zhi-Qing; Liu Ying-Bin; Han
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Medical University, Xi'an, Shanxi Province, China.

World journal of gastroenterology - WJG (China) Jul 2003 , 9 (7)
p1431-4, ISSN 1007-9327--Print Journal Code: 100883448

12) **ssociation of neurotrophin receptor expression and differentiation in human neuroblastoma.**

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13) **Expression of brain-derived neurotrophic factor and tyrosine kinase B receptor proteins in glioneuronal tumors from patients with intractable epilepsy: colocalization with N-methyl-D-aspartic acid receptor.**

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Association of Neurotrophin Receptor Expression and Differentiation in Human Neuroblastoma

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Interactions of the *trk* family of tyrosine kinase receptors with neurotrophins result in growth and maturational changes in neuronal cells. The continued progression, maturation, or regression of neuroblastoma, an embryonal, sympathetic nervous system-derived tumor of infants and children, might be governed by neurotrophic influences. Immunocytochemistry was utilized to evaluate TrkA, TrkB, and TrkC protein expression at the cellular level in the developing human fetal sympathetic nervous system and in a selection of neuroblastoma tumor specimens. TrkA and TrkC expression was identified in sympathetic ganglia and within the adrenal medulla, with intense TrkB expression restricted to paraganglia, of the normal developing human sympathetic nervous system. In neuroblastoma, pp140^{trkA} expression correlated positively with favorable tumor stage ($P = 0.0027$) and favorable outcome ($P = 0.026$). No statistically significant correlation of TrkC expression with outcome was evident. However, both TrkA and TrkC expression was most apparent in tumor cells of increased differentiation. TrkB expression was primarily localized to cells within the fibrovascular tumor stroma. A model of neurotrophin receptor expression and neurotrophin reactivity with differentiation is proposed. The existence and spatial distribution of neurotrophin receptors in neuroblastoma lend supportive evidence that neurotrophic influences may be involved in tumor persistence or regression. (Am J Pathol 1995, 147:102-113)

Nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin (NT)-3, and NT-4/5, the neurotrophin family of growth factors, promote differentiation, growth, and survival of central and peripheral nervous system neurons.¹⁻³ All members are synthesized as precursor polypeptides that, through enzymatic cleavage, yield mature homodimeric neurotrophins that differ in their sites of developmental expression and neuronal targets.³ Neurotrophic interactions with receptors of target neurons result in a variety of trophic and tropic events. NGF is recognized to promote survival and differentiation of neural crest sensory and sympathetic neurons among others.³ Brain-derived neurotrophic factor supports survival and outgrowth of a variety of central and neural crest-derived neurons but not sympathetic neurons. Both NT-3 and NT-4/5 have been described to act in a variety of neuronal locations.

The tyrosine kinase family of receptors encoded by the *trk* proto-oncogenes has been shown to be an essential component for neurotrophin interaction with neuronal cells.⁴⁻⁸ TrkA is the high affinity receptor for NGF, TrkB is the receptor for brain-derived neurotrophic factor and TrkC the receptor for NT-3. Some crossover exists as both TrkA and TrkB bind NT-3 and NT-4/5; however, they transduce their signals with lesser efficacy. Examination of the developing nervous system has confirmed the developmental regulation of the Trk receptors.⁹⁻¹³

Neuroblastoma (NB) is an embryonic, sympathetic nervous system-derived tumor of infancy and childhood, which may arise at any sympathetic nervous tissue location. Prognostic variability partly depends upon patient age at diagnosis and tumor stage, younger patients with less tumor burden enjoying the best prognosis.¹⁴⁻¹⁶ One intriguing feature of NB is

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that some disseminated tumors in infants may regress spontaneously even without treatment.^{17,18} Numerous investigations have sought tumor markers that might predict survival and guide therapy. *N-myc* amplification, degree of neuronal differentiation, mitosis-karyorrhexis index, *src* splicing variants, and levels of catecholamine metabolites are predictive of patient outcome.^{14,15,19-22} Recently, expression of insulin-like growth factor II mRNA and extent of cellular apoptosis have been shown to correlate inversely with disease aggressiveness; apoptosis is most apparent in tumor regions with increased cellular differentiation.^{23,24} Alterations in expression of the low affinity NGF receptor, p75^{NGFR}, have also been reported in clinical and cell culture NB materials.^{20,25} Similarly, low affinity NGF receptor and *trkA* mRNA expression have been shown to correlate with improved outcome in clinical tumors.^{20,25-27} In two cases in which primary NB cultures expressed *trkA*, NGF induced terminal differentiation whereas NGF deprivation resulted in cell death.²⁰

Because of the prognostic heterogeneity of NB with respect to stage of disease, apoptosis, and neurotrophin-directed maturation, we wished to further investigate high affinity neurotrophin receptor protein expression at the cellular level in NB and within the normal developing human sympathetic nervous system. We postulate that neurotrophin influences are involved in NB tumor cell maturation and predict that a loss of neurotrophin support could result in tumor cell death via apoptosis.

Materials and Methods

Tumor and Fetal Materials

Pertinent clinical features and materials were obtained from 36 patients registered and treated for NB (28), ganglioneuroblastoma (4), or ganglioneuroma (4) in Sweden over the past 7 years. Pathological materials from biopsy or resection of primary or metastatic tumor were evaluated and the diagnosis was confirmed at one of four referral hospitals in Göteborg, Lund, Stockholm, or Uppsala, Sweden. Because of the relative scarcity of low stage tumors, a representative selection of high stage tumors as well as all low stage tumors obtainable were studied. Tumor stage was determined clinically or at the time of surgical biopsy or resection according to the criteria of Evans et al.¹⁶ Patient characteristics were obtained from the treating clinicians. At a median follow-up of 31 months, all 10 patients diagnosed with stage I or stage II disease were alive and free of disease. Of the 10 patients with stage IV disease, 6 have died (60%),

1 patient remains alive with active disease, and 3 patients remain free of disease at a median follow-up of 29 months. *N-myc* amplification of greater than 10-fold was associated with 100% mortality (4/4). Patients were at varying stages of treatment at the time of tissue acquisition. The majority of patients with stage III and stage IV disease (60%) received cytotoxic therapy before the time of tumor biopsy or resection whereas those with stage I, II, and IVS had not (0%).

Human fetal tissue was obtained from both elective and spontaneous abortions from 9 to 25 weeks gestational age. Ethical approval (Dnr93-216) was obtained from the local ethical committee of Karolinska Hospital, Stockholm, Sweden.

Tissue Preparation and Immunocytochemistry

Tissue samples were fixed in 4% buffered formaldehyde and embedded in paraffin. Sections of 4 to 5 μ m were secured to slides pretreated with silane and acetone and deparaffinized. Sections were subjected to microwave treatment in 10 mmol/L sodium citrate buffer, pH 7.3, for 5 minutes at 750 watts and 10 minutes at 450 watts. Sections were then cooled in deionized distilled water (DDW) and blocked with 0.1% bovine serum albumin (BSA) in Tris-HCl-buffered saline for 20 minutes. Primary antibody at optimal dilution was added to sections with a 60-minute incubation at room temperature. Slides were rinsed three times with Tris-buffered saline buffer and incubated for 30 minutes at room temperature with alkaline phosphatase-conjugated monoclonal mouse anti-rabbit antibody (Dakopatts, Glostrup, Denmark) at 1:40 dilution. Sections were again washed and developed with Fast-red TR salt as chromogen according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). Slides were then rinsed in tap water for 10 minutes and counterstained with hematoxylin. Coverslips were applied in aqueous medium.

Antisera and Antisera Characteristics

Primary antibodies included polyclonal rabbit Trk (TrkA immunoglobulin (763); Santa Cruz Biotechnology, Santa Cruz, CA), epitope corresponding to amino acids 763 to 777 mapping adjacent to the carboxy terminus of human *trk* p140 (1:100 dilution); polyclonal rabbit TrkB immunoglobulin (794; Santa Cruz Biotechnology), epitope corresponding to amino acids 794 to 808 mapping adjacent to the carboxy terminus of human *trkB* p145 (1:100 dilution);

truncated polyclonal rabbit TrkB immunoglobulin (C-13; Santa Cruz Biotechnology), epitope corresponding to amino acids mapping at the carboxy terminus of human truncated *trkB* p95 (1:100 dilution); and polyclonal rabbit TrkC immunoglobulin (798; Santa Cruz Biotechnology), epitope corresponding to amino acids 798 to 812 mapping adjacent to the carboxy terminus of porcine *trkC* p140 (1:100 dilution). Additionally, anti-Trk antisera corresponding to both intracellular and extracellular domains of TrkA, TrkB, and TrkC proteins (TrkAin, TrkAout, TrkBin, TrkBout, TrkCin, TrkCout, and panTrk)²⁸⁻³⁰ were investigated. Optimal antibody concentration as determined by serial dilutions was used in all instances. Equivalent immunoreactivity was obtained in paraffin-embedded and in similarly prepared snap-frozen sections of corresponding tumors. Negative controls were obtained by exclusion of primary antibody or by incubation with nonimmune bovine serum albumin at equivalent concentrations. Preincubation of the anti-TrkA, anti-TrkB, and anti-TrkC antisera with the corresponding peptides used for immunization (Santa Cruz Biotechnology) resulted in complete loss of specific immunoreactivity for respective antisera. Cross-incubation of these peptides to the antisera, eg, TrkC control peptide addition to anti-TrkA antisera incubations, did not affect the specific immunocytochemical staining.

To further characterize the antisera used, Western blot analyses of cell lysates from Sf9 insect cells infected with recombinant baculoviruses expressing TrkA,³¹ TrkB (RM Stephens, D Soppet, L Parada, DR Kaplan, unpublished observations), or TrkC (P Tsoulfas, L Parada, unpublished observations) were performed. Cell lines were provided by Dr. Anna Maroney and Dr. Craig Dionne (Cephalon, West Chester, PA). Cell lysates were prepared as previously described,³² electrophoresed on 7.5% sodium dodecyl sulfate polyacrylamide gels, transferred to nitrocellulose filters, probed with the above described Trk antibodies,³² and developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Antisera specificities were also investigated by using NIH-3T3 cells specifically transfected with TrkA, TrkB, or TrkC, grown to semiconfluence, washed with phosphate-buffered saline (PBS), fixed for 10 minutes in methanol, and subjected to the immunocytochemical protocol as above.

Immunocytochemistry utilizing anti-neuron-specific enolase (NSE) IgG antiserum (Dakopatts) at 1:50 dilution, anti-S100 IgG antiserum (Dakopatts) at 1:100 dilution, and anti-chromogranin A&B IgG antiserum (Dakopatts) at 1:2000 dilution was performed in similar fashion.

DNA Nick End-Labeling of Tissue Sections

A procedure to specifically end label DNA cleavage sites in tissue sections *in situ* (TUNEL) was employed as previously described with minor modifications.³³ Paraffin-embedded sections were deparaffinized and incubated in a moist chamber for 15 minutes with 20 µg/ml proteinase K (Sigma Chemical Co.) and thereafter washed three times with DDW. Two percent hydrogen peroxide was added for 5 minutes to inactivate endogenous peroxidase, and the sections were again washed three times with DDW. Each section was then incubated at 37°C for 60 minutes with terminal deoxynucleotidyl transferase (10 enzymatic units/50 µl) and biotinylated deoxyuridine (dUTP, 0.5 nmol/50 µl; both Boehringer Mannheim, Mannheim, Germany) in transferase buffer (30 mmol/L Tris-HCl buffer, pH 7.2, 140 mmol/L sodium cacodylate, and 1 mmol/L cobalt chloride). Reaction was terminated by immersion in 300 mmol/L NaCl and 30 mmol/L sodium citrate buffer for 15 minutes. Sections were washed three times with DDW, blocked for 10 minutes with 2% bovine serum albumin at room temperature, and washed three times again with DDW. Sections were immersed in PBS for 5 minutes and then incubated with avidin-biotin complex (1:100 avidin and 1:100 biotinylated horseradish peroxidase in 0.1% bovine serum albumin; Dakopatts) for 30 minutes according to the manufacturer's instructions. Sections were washed three times with DDW, immersed in PBS for 5 minutes, developed in 3-amino-9-ethylcarbazole solution for 20 minutes at room temperature, and washed for 10 minutes in DDW. Coverslips were applied in aqueous medium.

Quantitation of Trk Expression

A semiquantitative scale to grade the degree of Trk immunoreactivity was used in all NB specimens. A scale from 0 to 5 was used: 0, no staining; 1, trace immunoreactivity evident; 2, occasional cells stain; 3, minority of cells stain; 4, majority of cells stain; and 5, all cells stain intensely. Fifteen randomly selected regions of each specimen were examined under high power, each region was rated, and the median rating of the fifteen regions was determined. Care was taken to avoid examining regions with apparent necrosis. Consistent results were obtained between repeat experiments and between different block sections of tumor tissue from the same patient. Standardized specimens were analyzed with each assay to ensure reproducibility of the scoring scale. Immunocytological examination was blinded with respect to patient outcome and tumor stage. The mean and standard deviation of immunocytochemical staining grade for

all tumors stratified according to tumor stage and outcome was calculated, and statistical comparison performed by Student's *t*-test.

Results

Anti-Trk Antisera Specificities

Three sets of anti-Trk antisera were used, one commercially available and the other two generated by independent research groups. As mentioned below, and exemplified in Figure 1, these antisera gave equivalent results in normal fetal tissue and in tumor sections but with somewhat increased nonspecific background reactivity for some antisera when staining tumor sections. Identical results were obtained comparing the differing antisera for a given protein in other neuron-harboring tissues, ie, sections of human fetal and postnatal intestine (JC Hoehner, T Wester, D Kaplan, S Pålman, L Olsen, manuscript in preparation).

The similar immunocytochemical reactivity patterns resulting from anti-TrkA and anti-TrkC antisera prompted a more careful characterization of the specificities of these antisera to rule out cross-reactivity between anti-TrkC antisera and TrkA and vice versa. To that end, cell lysates from TrkA-, TrkB-, and TrkC-overexpressing Sf9 cells were analyzed by Western blotting with those antisera that perform well in Western protocols. The anti-TrkA (763) antisera, as well as all other specific, noncommercial antisera tested, recognized one major protein with the expected molecular weight, in the respective transfectant (Figure 2A and data not shown).

The antisera specificities were similarly tested in the immunohistochemical protocol with Trk-overexpressing NIH-3T3 cells. Immunoreactive positivity was identified only in transfected cells with the respective specific appropriate antisera. In particular, the anti-TrkC antiserum (798) did not stain the TrkA-overexpressing cells, and the anti-TrkA antiserum (763) did not stain the TrkC-transfected cells (Figure 2, B and C). Thus, no cross-reactivity of specific antibodies to differing Trk-overexpressing cells was identified in any of the three cell lines when tested with all above listed antisera. Furthermore, antisera directed to the same Trk species gave equivalent staining results both in normal and in tumor tissues; however, slight differences in background staining were evident, and the best results were obtained with the anti-TrkA (763) and anti-TrkB (794) antisera.

Neurotrophin Receptor Expression in Human Fetal Materials

Results of TrkA, TrkB, and TrkC immunoreactivities at human fetal gestational ages 9 to 24 weeks are summarized in Table 1. In general, immunoreactivity increased in tissues of the developing sympathetic nervous system with increasing gestational ages. Neuronal cells of dorsal root ganglia, pre-aortic sympathetic ganglia, and sympathetic trunk ganglia stained intensely for both TrkA and TrkC in the 24-week gestational age fetus with lesser but identifiable immunoreactivity at earlier ages (Figure 3). Mature neuronal cells with pale nuclei and generous cytoplasm stained most intensely. These cells exist in

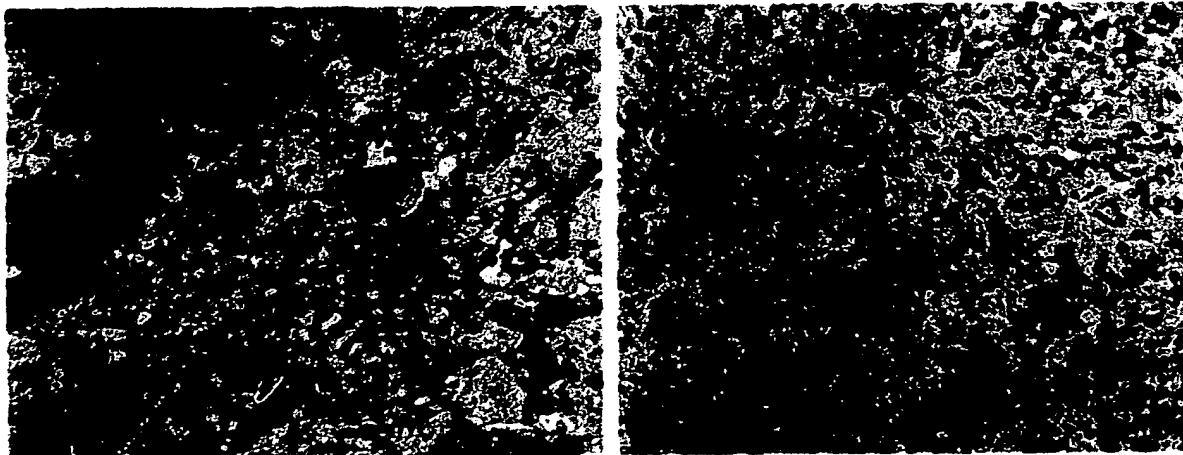


Figure 1. Characterization of anti-Trk antisera immunoreactivity in clinical NB tumors. Sections of a stage II NB tumor immunocytochemically stained with anti-TrkA (763) antiserum in (A) and anti-TrkAin antiserum in (B). Immunoreactive positive cells are indicated by red cytoplasmic staining. Intense positivity detected with both antisera in cytoplasmic-rich, differentiated tumor cells (arrowheads). Differentiated tumor cells forming pseudorosettes are also immunoreactively positive with both antisera (arrows). Less well differentiated groups of tumor cells with dense nuclei and scant cytoplasm lack or show very little Trk immunoreactivity. Scale bar, 200 μ m.

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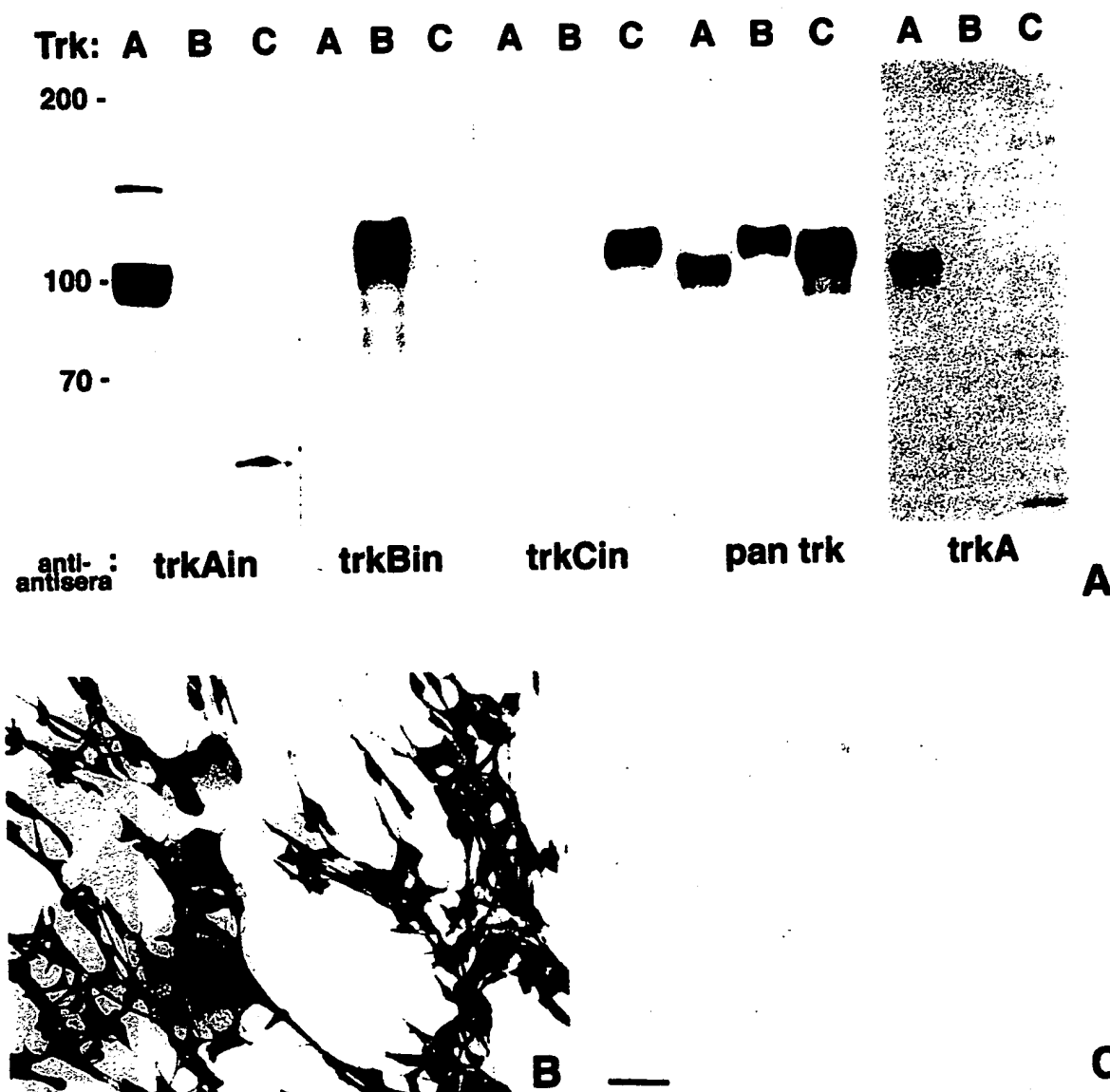


Figure 2. Characterization of anti-Trk antisera. **A:** Western blot analysis of cell lysates of Sf9 cells overexpressing either TrkA (A), TrkB (B), or TrkC (C) was performed with specific anti-Trk antisera as probes. Shown are five identical Western blots prepared from the indicated Trk-overexpressing cultures shown at top. Molecular weight markers in kilodaltons are indicated at left. Note single band at appropriate molecular weight detected in each specific Trk clone by corresponding antisera. No crossover recognition of anti-Trk antisera to other Trk species was evident. **B:** Immunocytochemistry of NIH-3T3 TrkA-overexpressing cells stained with anti-TrkA (763) antiserum. Dark cytoplasmic staining of cells indicates immunopositivity. **C:** Immunocytochemistry of NIH-3T3 TrkA-overexpressing cells stained with anti-TrkC (798) antiserum. No immunoreactivity was detected. Scale bar, 50 μ m.

greater abundance at later gestational ages. Poorly differentiated sympathetic neuronal cells stained less intensely for TrkA and TrkC, with diminished immunoreactivity at earlier gestational ages. In these tissues, TrkB immunoreactivity ranged from nonexistent to barely discernible (Table 1). Both chromaffin cells and primitive neuronal cells within the adrenal medulla revealed detectable but less intense immunoreactivity with TrkA and TrkC. Paraganglia cells of the organ of Zuckerkandl exhibited intense anti-TrkB antiserum immunoreactivity only at later gestational

ages (Table 1). Similarly, only trace staining of mature peripheral nerve trunks was apparent for TrkA and TrkB. Immunoreactivity of the truncated form of TrkB was similar to that of TrkB with the exception of enhanced smooth muscle reactivity. Immunoreactivity with pan-Trk antibody confirmed staining as the sum of all three Trk immunoreactivities. Other fetal tissues also stained positively with anti-Trk antisera, specifically liver and peripheral macrophages with TrkB and pancreatic islet cells with TrkA and TrkB antisera (data not shown).

Table 1. Neurotrophin Receptor Expression in Human Fetal Peripheral Nervous System

	Paraganglia	DR ganglia	Sympathetic ganglia	Nerve trunks	Adr medulla	Adr neuronal
TrkA						
8-9 weeks		Trace	Trace	Trace		
13-15 weeks	+	+	+	Trace	Trace	+
21-24 weeks	++	++	++	Trace	Trace	+
TrkB						
8-9 weeks		-	-	-		
13-15 weeks	Trace	-	-	Trace	-	-
21-24 weeks	++	Trace	Trace	Trace	Trace	-
TrkC						
8-9 weeks		Trace	-	-		+
13-15 weeks	+	+	+	-	+	+
21-24 weeks	++	++	++	-	+	+

Expression of TrkA, TrkB, and TrkC in 8-9, 13-15, and 21-24-week gestational age human fetuses confined to paraganglia, dorsal root ganglia (DR ganglia), sympathetic ganglia, peripheral nerve trunks, adrenal medullary chromaffin cells (Adr medulla), and primitive neuronal cells of the adrenal medulla (Adr neuronal). (-) indicates absent immunoreactivity; (+), immunoreactive positivity; (++), intense immunoreactivity; and Trace, trace immunoreactivity.

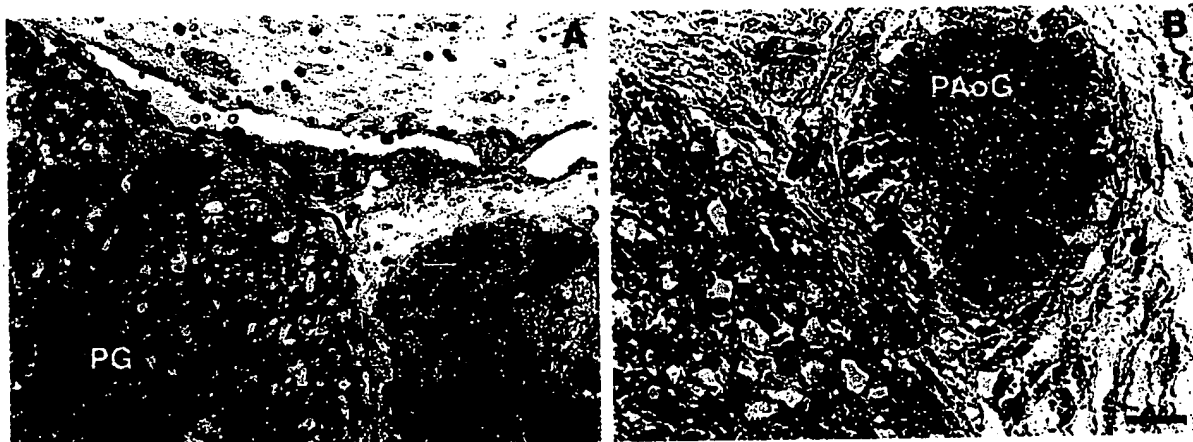


Figure 3. TrkA immunoreactivity in human fetal tissues. A: Abdominal cross section through pre-aortic region of 25-week gestational age fetus. Immunoreactivity (763 antiserum) represented by cytoplasmic staining of pre-aortic sympathetic ganglia (PAoG), and paraganglia (PG). B: High power photomicrograph of pre-aortic area of 25-week gestational age fetus. Immunoreactivity is most evident in sympathetic neuroblasts with abundant cytoplasm and pale nuclei. Staining of paraganglia also evident. Counterstaining was not performed. Scale bar, 200 μ m (A) 25 μ m (B).

TrkA Expression in Neuroblastoma

All tumors of the 29 infants and children with NB, without exception, stained positively for TrkA to varying degrees (Figure 4, A and B). Staining was both membranous and cytoplasmic, without evidence of nuclear staining, and specific, as revealed by blocking the immunoreactivity by antisera preincubation with the corresponding immunization peptide (data not shown). Equivalent TrkA staining was observed in identical snap-frozen specimens, fixed in acetone, compared with formalin-fixed paraffin-embedded specimens. Only neuroblastoma cells proper stained positively; the stroma and/or fibrous aspects of the tissue did not stain with TrkA antisera. All NB specimens exhibited heterogeneity with respect to degree of cellular differentiation. Differentiated neuroblasts with a low nuclear to cytoplasmic ratio and pale nuclei stained most intensely for TrkA (Figures 1 and 4, A and B). Less mature cells, with densely hematoxylin-

stained nuclei and little cytoplasm, stained less intensely. In tumors without a well developed fibrovascular supportive stroma, the anti-TrkA antiserum stained neuroblastoma cells in a random, scattered pattern. In two tested cases with N-myc amplification, TrkA immunoreactivity was apparent in both tumor specimens. TrkA expression in the morphologically more mature cells of ganglioneuroblastoma and ganglioneuroma was similar to that of NB, with staining most apparent in mature, differentiated neuronal cells (Figure 5A).

TrkB Expression in Neuroblastoma

Expression of TrkB protein in our selection of NB was detected within the supportive fibrovascular stromal elements of the tumor in a scattered pattern (Figure 4C). Immunoreactivity with an antiserum directed towards the truncated form of TrkB was not appreciably different from

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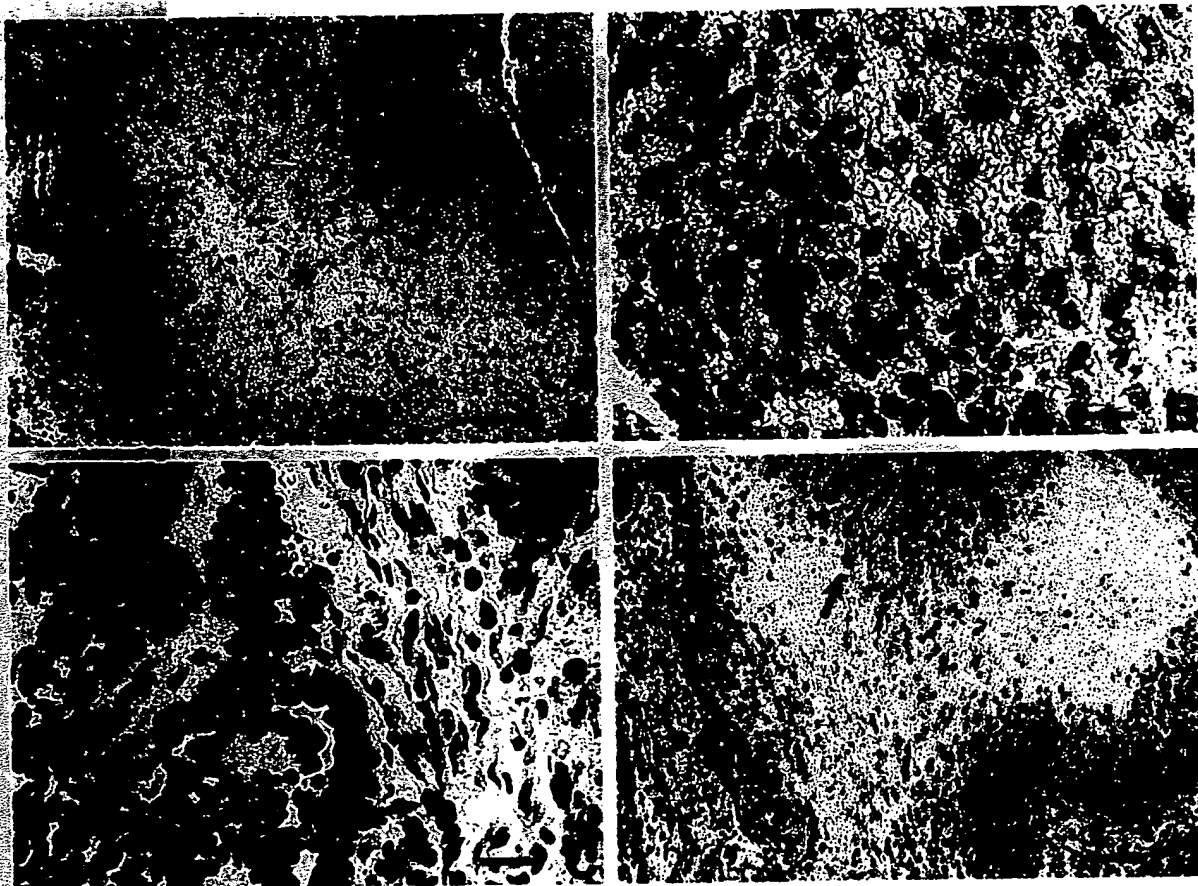


Figure 4. Trk immunoreactivity in NB. A: Photomicrograph of an anti-TrkA-stained stage II NB. Note thin, well developed fibrovascular stroma (arrows), zone of malignant neuroblasts, and central zone of cellular remnants (*). Red anti-TrkA (763 antiserum) immunoreactivity is most intense in differentiated neuroblasts lying adjacent to central zone. B: High power photomicrograph of anti-TrkA-stained specimen (A), revealing minimal immunoreactivity in neuroblasts adjacent to peritubular fibrovascular stroma (arrow), with intense immunoreactivity in differentiated neuroblasts with pale nuclei and abundant cytoplasm adjacent to central zone (*). C: Photomicrograph of stage II NB stained with anti-TrkB (794) antiserum. Note intense red immunoreactivity in spindle cells of fibrovascular stroma (arrow) and absent immunoreactivity in primitive neuroblasts proper (left). D: Photomicrograph of stage IVS NB stained for TrkC (798 antiserum). Note centralized dumbbell-shaped zone of cellular remnant adjacent to differentiated immunoreactive neuroblasts (arrow), bounded by peripheral tumor stroma. Scale bar, 200 μ m (A and D) 35 μ m (B and C).

that of the anti-TrkB antiserum. Immunoreactive positive cells within fibrovascular stroma morphologically resembled macrophages, fibroblasts, or Schwann cells. TrkB immunoreactivity mirrored that of the Schwann cell marker S100 (not shown). In general, fibrovascular stroma-rich tumors displayed greater TrkB immunoreactivity than stroma-poor tumors, with the majority of immunoreactivity within the non-tumor-cell compartments of the tissue. Immunoreactivity of neuroblastic tumor cells proper was confirmed in only one fatal N-myc-amplified stage IV tumor. No staining correlation with respect to extent of cellular differentiation was identified with anti-TrkB antiserum.

TrkC Expression in Neuroblastoma

As with TrkA immunoreactivity, all tumors examined displayed TrkC immunoreactivity to varying degrees. Staining was specific and appeared to be both cy-

toplasmic and membranous. (Figure 4D) Neuroblastic cells stained most intensely, with minimal evidence of staining within stromal supportive elements. As in the TrkA stainings, neuroblasts with a low nuclear to cytoplasmic ratio and pale nuclei, more highly differentiated tumor cells, stained most intensely. (Figure 5B) Morphologically less mature neuroblasts displayed significantly less immunoreactivity. In tissues with a disorganized arrangement lacking a well developed fibrovascular stroma, immunoreactive cells were randomly scattered throughout. As with TrkA, ganglioneuroblastoma and ganglioneuroma TrkC immunoreactivity was most intense in more mature, differentiated neuronal cells.

Quantitation of Trk Expression

Statistically significantly increased TrkA expression was apparent in tumor specimens of patients with im-

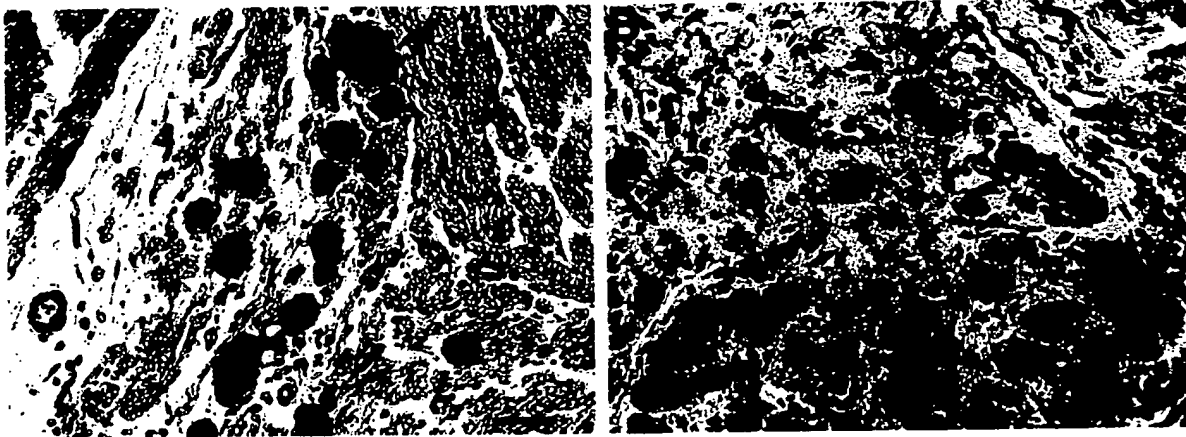


Figure 5. Trk immunoreactivity correlation with maturation. A: Anti-TrkA (763 antiserum)-stained ganglioneuroma tumor. Note intense cytoplasmic immunoreactivity of large cytoplasmic-rich, mature neuronal cells (arrow), with absent staining of surrounding supportive elements. B: Anti-TrkC (798 antiserum)-stained photomicrograph of stage IV NB. Immunoreactivity is most evident in ganglia-like, mature, differentiated neuroblasts with abundant cytoplasm and pale nuclei (arrow). Immature neuroblastic cells and supportive elements are TrkC negative. Counterstained with hematoxylin; scale bar, 70 μ m (A), 35 μ m (B).

proved outcome (2.56 ± 0.80 for survivors *versus* 1.91 ± 0.29 for nonsurvivors; $P = 0.026$), and with favorable tumor stage (3.0 ± 0.91 for stages I, II, and IVS *versus* 1.88 ± 0.48 for Stages III and IV; $P = 0.0027$). The semiquantitative analysis of TrkA expression is represented in Figure 6. A similar trend was seen for TrkC immunoreactivity, but statistical significance was not achieved ($P = 0.08$). No statistical differences were identified for TrkB immunostaining stratified according to outcome or tumor stage ($P > 0.05$).

NB Lobule: TrkA and TrkC Expression and Maturation

In NB tissue with more organized histology, primarily low stage tumors, a specific pattern of TrkA and TrkC immunoreactivity was evident. In tumor sections with a lobular or acinar arrangement of tumor cells, TrkA and TrkC expression was least evident in poorly differentiated neuroblasts adjacent to the perilobular stroma, capillaries, and blood vessels (Figure 4). These cells typically displayed an increased nuclear to cytoplasmic ratio with dense nuclei and scant cytoplasm. TrkA and TrkC expression progressively increased in cells nearing the center of the lobule structure, near that of the central cellular remnant zone (Figure 7). Cells about this cellular remnant zone have pyknotic and condensed nuclei, with fragmented DNA as demonstrated by the TUNEL technique, suggestive of an apoptotic cellular death process (Figure 8). Cells expressing TrkA and TrkC displayed pale nuclei and more abundant cytoplasm than those small, undifferentiated cells lying adjacent to the stroma and capillaries. Similarly, NSE and chro-

mogranin A&B immunoreactivity, as well as hematoxylin and eosin morphology, confirmed increasing cellular maturation in neuroblasts nearing the lobule center (not shown).

Discussion

Our investigation included an examination of human fetal tissue in conjunction with that of NB because of the known and presumed prenatal generation of NB, the prognostic dichotomy of NB relative to age of diagnosis, a lack of understanding of NB progenitor cell(s), and the known existence of spontaneous regression in NB. Furthermore, the potential importance of apoptosis in both NB and within the developing sympathetic nervous system lead us to assume that fetal tissue characteristics are important in evaluating the generation of NB.

Trk immunoreactivity was evident within the developing human sympathetic nervous system. Paraganglia, pre-aortic ganglia, and sympathetic trunk ganglia, the presumptive progenitors of neuroblastoma, stained intensely with TrkA and TrkC at later gestational ages. In these structures, immunoreactivity to all Trk antibodies increased with increasing gestational age. The adrenal medulla, consisting of both chromaffin and primitive sympathetic neuronal cells at the developmental stages studied, revealed detectable but less intense TrkA and TrkC immunoreactivity. Sensory dorsal root ganglia stained with all Trk antibodies but only minimally so for TrkB. Interestingly, at the latest gestational ages, intense TrkB immunoreactivity was localized only to paraganglia.

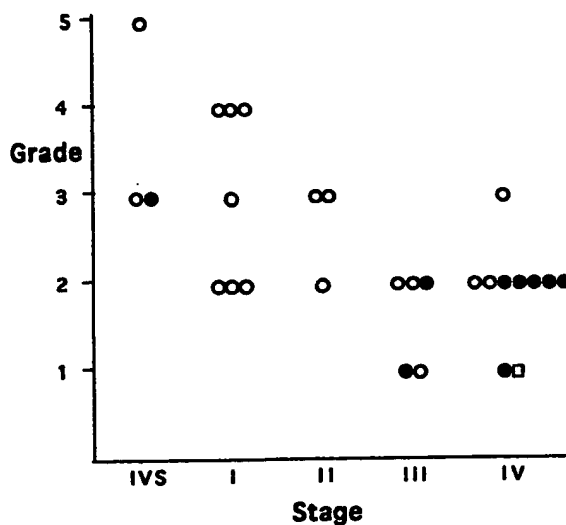


Figure 6. Semiquantitative grading of TrkA expression in NB. A: Graphic representation of TrkA immunoreactivity grade (ordinate) in NB stratified according to tumor stage (abscissa). Open circles (○) denote long-term survivors, filled circles (●) denote nonsurvivors, and open square (□) denotes alive with active disease. The single death in the stage IVS group occurred as a result of neuroendocrine crisis rather than tumor burden. Note trend of diminishing staining grade with tumor stages of worsened prognosis. Statistical comparisons between survivors and nonsurvivors by Student's t-test reveals statistically significant increased TrkA staining in survivors versus nonsurvivors ($P = 0.026$).

With the presumptive sites of NB being that as mentioned above, altered TrkA and TrkC signaling in these tissue types could influence malignant cell growth.

In NB tissues, immunoreactivity of tumor cells proper was most evident with TrkA and TrkC antisera. In accordance with *trkA* expression previously reported at the mRNA level,^{20,25-27} we similarly found increased TrkA protein expression in favorable outcome, low stage tumors. Neither TrkB nor TrkC expression grade conclusively correlated with prognosis or tumor stage. Therefore, the possibility that NGF via TrkA responsiveness directs NB tumor growth is feasible. Others have shown that *N-myc* amplification, extent of neuronal differentiation, and the mitosis karyorrhexis index correlate with tumor stage and prognosis.^{14,19-21} Previously, correlations of the degree of apoptosis, maturation, and expression of the death suppressor proto-oncogene *bcl-2* with favorable outcome have been reported.^{24,34} The current study suggests that TrkA and TrkC expression also vary with neuronal differentiation. Within a given tumor, differential degrees of cellular maturation often exist. In those tumor cells of immature morphology, both TrkA and TrkC expression was low. We found increased expression of both TrkA and TrkC in more mature tumor cells, as evidenced by morphology, and NSE and chromogranin A&B immunoreactivity. This

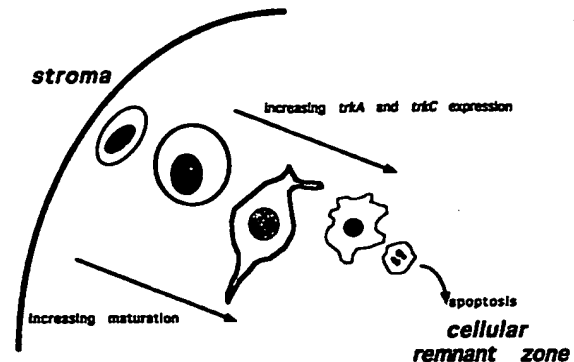


Figure 7. Neuroblastoma lobule: maturation and Trk expression. Representation of NB tumor lobule as described in text. Low TrkA and TrkC expression exists in neuroblasts adjacent to tumor stroma. An increase in TrkA and TrkC expression occurs with increasing maturation as neuroblasts locate toward central cellular remnant zone. Cellular death by apoptosis occurs at the center of the lobule structure.

spatial arrangement of maturation and Trk expression is most apparent in lower stage tumors (stages I, II, and IVS) with improved prognosis in contrast to those with worsened prognosis (stages III and IV).

Recently, expression of *trkB* mRNA in homogenates of poor prognosis high stage NB has been reported, with truncated *trkB* expression found primarily in benign ganglioneuroblastoma and ganglioneuroma.³⁵ Similarly, retinoic acid-induced NB cell cultures also express full length and truncated *trkB*.²⁸ We report no prognostic dependency of TrkB expression in our limited NB material; however, TrkB expression was identified in non-neuroblastic cells lying within fibrovascular stroma and in neuroblastic cells of a single *N-myc*-amplified tumor. This finding that TrkB immunoreactivity exists within fibrovascular stroma cells may indicate that tumor stromal cells and hence tumor angiogenesis through paracrine interactions are dependent upon this form of neurotrophic support. Our limited number of *N-myc* amplified tumors available make conclusions regarding TrkB expression in this tumor form irrelevant; however, our single case with neuroblastic TrkB positivity is consistent with the reported TrkB expression in some NB cell lines and tumors with *N-myc* amplification.^{27,34}

The maturational spatial arrangement of Trk expression that we describe may provide an explanation for the biological activity of these tumors. In NB tumors of lesser stage, neuroblastic cells residing adjacent to the fibrovascular stroma are poorly differentiated, lacking Trk expression. As these primitive cells presumably locate away from this stroma, they mature morphologically with subsequent increased chromogranin A&B and NSE expression. We also find

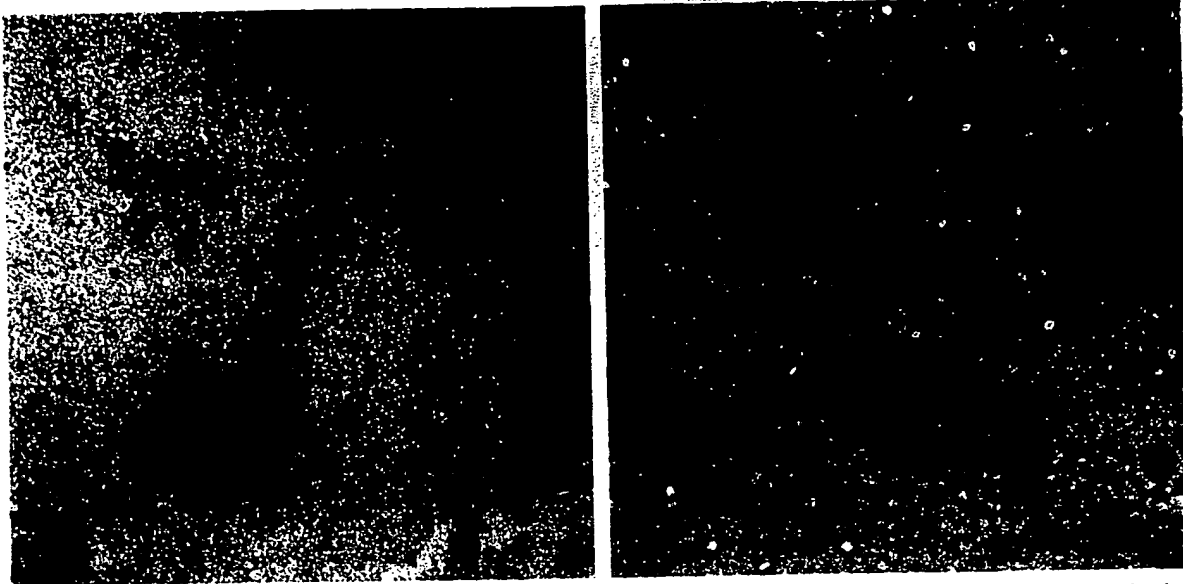


Figure 8. Detection of apoptosis in NB. **A:** Photomicrograph of stage II NB stained by TUNEL technique. Fibrovascular stroma margins of a characteristic tumor lobule demarcated by black arrows. Lobule center of cellular remnants reveals adjacent scattered nuclear cellular staining (arrowhead). Minimal nuclear staining is apparent in the majority of neuroblasts and about tumor stroma. **B:** High power view of (A) of a large group of apoptotic cells adjacent to cellular remnant zone. Note intense, nuclear specific staining of apoptotic neuroblasts (arrow), the majority of which reveal nuclear condensation. Cells with fragmented nuclei (black arrowhead) as well as apoptotic bodies (open arrowhead) are also TUNEL positive. Scale bar, 250 μ m (A), 25 μ m (B).

that other tumor cells located adjacent to these differentiated cells are pyknotic, with condensed nuclei and fragmented DNA suggestive of apoptotic cell death. With differentiation *in situ*, TrkA and TrkC expression increase, or the corollary, as Trk expression increases, the cells undergo differentiation. This might suggest that, as NB cells acquire increasing degrees of differentiation, they develop an increased capability to respond to neurotrophins by increased receptor expression. Whether this occurs is uncertain; however, one study suggests that tumor cells acquired from good prognosis, TrkA-expressing tumors, do differentiate in response to NGF, with NGF deprivation resulting in cellular death.²⁰ It is not known whether the same is true of tumors that express TrkB or TrkC. This might suggest that a deficiency in a circulation-derived substance, such as a metabolic substrate or growth factor, or relative ischemia may regulate Trk expression and incite cellular differentiation. Tumors with high Trk expression do show higher levels of the differentiation markers NSE, synaptophysin, and neuronal c-src protein³⁶ and, therefore, increased expression of the neurotrophin receptors in good prognosis tumors may merely be a reflection of a more differentiated and therefore less aggressive state.

An obvious shortcoming of this study is the inability to acquire tumor specimens before chemotherapeu-

tic treatment in all patients with high stage tumors. Others have indicated that various chemotherapeutics exert their effect by induction of apoptosis and cellular death mechanisms,³⁷ suggesting that these agents might act through a type of cellular maturation pathway as well. It is conceivable that Trk expression alterations could occur in those tumors acquired after chemotherapeutic treatment; however, the histological features of the neuroblastoma lobule we describe should remain unaltered as this pattern is most apparent in nontreated low stage tumors and persists in tumor specimens obtained both before and after treatment.

The question regarding Trk expression and neurotrophin responsiveness in NB has become an important one. It is known that most NB cell lines are trophic and tropic unresponsive to NGF.^{19,38} The majority of these cell lines, however, are derived from high stage, aggressive, N-myc-amplified tumors.¹⁹ A variety of NB cell cultures nevertheless do express *trk* and the low affinity NGF receptor p75 in lieu of their NGF unresponsiveness. In fact, when cultured NB cells are induced to undergo differentiation by a variety of means, Trk expression increases but with continued NGF unresponsiveness.³⁹ NB cell lines exogenously transfected with *trk* do become NGF responsive with resultant growth and differentiation, even though these cells express endogenous *trk* before transfec-

tion. High stage NB may be NGF or neurotrophin unresponsive either by a gene dose-related phenomenon whereby cellular receptivity to neurotrophins is diminished or possibly by structural defects in endogenous p75^{NGFR} or Trk.

We postulate that in low stage NB, TrkA or TrkC responsiveness may play a role in persistent tumor growth or regression. In contrast, higher stage tumors maintain a less effective mechanism of neurotrophin reactivity, at least with respect to TrkA and the neurotrophin NGF. Others have described coexistence of both the neurotrophins and their receptors in the developing nervous system and confirmed responsiveness and expression of the neurotrophins within the same tissue.^{9,13} Investigations to determine whether neurotrophin activity exists within the neuroblastoma tissue or whether neurotrophins are blood borne or endogenously produced by the tumor cells or stroma have not been successful thus far. Neurotrophic influences might also be important in the cellular maturation and/or subsequent self-destruction of NB; ie, a lack of neurotrophin support may result in cellular death. Ultimately, the potential to potentiate neurotrophic influences in NB tumors with consequent induction of differentiation may also prove to encourage cellular death via apoptosis.

Acknowledgments

We thank Anna Maroney and Craig Dionne (Cephalon, West Chester, PA) for supplying the cell lines used in this work and Pantelis Tsoulfas and Luis Parada for the TrkC baculovirus.

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File 155:MEDLINE(R) 1950-2006/Oct 09

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File 55:Biosis Previews(R) 1993-2006/Oct W1

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File 34:SciSearch(R) Cited Ref Sci 1990-2006/Oct W1

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File 434:SciSearch(R) Cited Ref Sci 1974-1989/Dec

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File 340:CLAIMS(R)/US Patent 1950-06/Oct 05

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***File 340: IPCR/8 classification codes now searchable in 2006 records.**

For important information about IC=index changes, see HELP NEWSIPCR.

Set	Items	Description
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? s trkb		
S1	6358	TRKB
? s neurotrophin? or NT4 or (NT(w)4)		
Processing		
	62786	NEUROTROPHIN?
	538	NT4
	49451	NT
	9837792	4
	2002	NT(W)4
S2	63166	NEUROTROPHIN? OR NT4 OR (NT(W)4)
? s cancer? or tumor? or malignan?		
Processing		
	1742492	CANCER?
	2094993	TUMOR?
	630722	MALIGNAN?
S3	3432215	CANCER? OR TUMOR? OR MALIGNAN?
? s s1 and s3		
	6358	S1
	3432215	S3
S4	609	S1 AND S3
? s s4 and py<=2002		
Processing		
Processing		
	609	S4
	44693562	PY<=2002
S5	453	S4 AND PY<=2002
? s s5 and s2		
	453	S5
	63166	S2
S6	224	S5 AND S2
? t s6 and py<2002		
>>>'AND' not allowed in command		
? rd		

>>>Duplicate detection is not supported for File 340.

>>>Records from unsupported files will be retained in the RD set.

S7 138 RD (unique items)

? s s7 and py<2002

Processing

138 S7

42205159 PY<2002

S8 122 S7 AND PY<2002

? s (NT(w)4) or NT4?

49451 NT
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 2002 NT(W)4
 618 NT4?
 S9 2552 (NT(W)4) OR NT4?
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Set	Items	Description
S1	6358	TRKB
S2	63166	NEUROTROPHI? OR NT4 OR (NT(W)4)
S3	3432215	CANCER? OR TUMOR? OR MALIGNAN?
S4	609	S1 AND S3
S5	453	S4 AND PY<=2002
S6	224	S5 AND S2
S7	138	RD (unique items)
S8	122	S7 AND PY<2002
S9	2552	(NT(W)4) OR NT4?

? s s1 and s9
 6358 S1
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 ? s s10 and s3
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 S11 34 S10 AND S3
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>>>Records from unsupported files will be retained in the RD set.

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 Processing

23 S12
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 S13 17 S12 AND PY<=2002
 ? t s13/3,k,ab/1-17

13/3,K,AB/1 (Item 1 from file: 155)
 DIALOG(R)File 155:MEDLINE(R)
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13408110 PMID: 11583980

Differential cellular expression of neurotrophins in cortical tubers of the tuberous sclerosis complex.

Kyin R; Hua Y; Baybis M; Scheithauer B; Kolson D; Uhlmann E; Gutmann D; Crino P B

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American journal of pathology (United States) Oct 2001 , 159 (4)
 p1541-54, ISSN 0002-9440--Print Journal Code: 0370502

Contract/Grant No.: K08-01658; PHS

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Neurotrophins and their receptors modulate cerebral cortical development. Tubers in the tuberous sclerosis complex (TSC) are characterized

histologically by disorganized cortical cytoarchitecture and thus, we hypothesized that expression of neurotrophin mRNAs and proteins might be altered in tubers. Using in situ transcription and mRNA amplification to probe cDNA arrays, we found that neurotrophin-3 (NT3) and **trkB** mRNA expression were reduced whereas neurotrophin-4 (**NT4**) and **trkC** mRNA expression were increased in whole tuber sections. Alterations in mRNA abundance were defined in single microdissected dysplastic neurons (DNs) and giant cells (GCs). NT3 mRNA expression was reduced in GCs and **trkB** mRNA expression was reduced in DNs. **NT4** mRNA expression was increased in DNs and **trkC** mRNA expression was increased in both DNs and GCs. In three patients, TSC2 locus mutations were confirmed and the mean tuberin mRNA expression levels was reduced across all nine cases. Consistent with these observations, NT3 mRNA expression was reduced but **trkC** mRNA expression was increased in vitro in human NTera2 neurons (NT2N) transfected with a tuberin antisense construct that reduced tuberin expression. Western analysis of tuber homogenates and computer-assisted densitometry of immunolabeled sections confirmed the neurotrophin mRNA expression data in whole sections and single neurotrophin immunoreactive cells. We conclude that alterations in **NT4** / **trkB** and NT3/**trkC** expression may contribute to tuber formation during brain development as downstream effects of the hamartin and tuberin pathway in TSC.

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... transcription and mRNA amplification to probe cDNA arrays, we found that neurotrophin-3 (NT3) and **trkB** mRNA expression were reduced whereas neurotrophin-4 (**NT4**) and **trkC** mRNA expression were increased in whole tuber sections. Alterations in mRNA abundance were...

... dysplastic neurons (DNs) and giant cells (GCs). NT3 mRNA expression was reduced in GCs and **trkB** mRNA expression was reduced in DNs. **NT4** mRNA expression was increased in DNs and **trkC** mRNA expression was increased in both DNs...

...expression data in whole sections and single neurotrophin immunoreactive cells. We conclude that alterations in **NT4** / **trkB** and NT3/**trkC** expression may contribute to tuber formation during brain development as downstream effects...

...; t; Research Support, U.S. Gov't, P.H.S.; Transfection; Tuberous Sclerosis--genetics--GE; **Tumor** Suppressor Proteins

Chemical Name: Antisense Elements (Genetics); Nerve Growth Factors; RNA, Messenger; Repressor Proteins; **Tumor** Suppressor Proteins; tuberous sclerosis 2 protein

13/3,K,AB/2 (Item 2 from file: 155)

DIALOG(R)File 155:MEDLINE(R)

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13388105 PMID: 11561004

Expression of nerve growth factors in pancreatic neural tissue and pancreatic cancer .

Schneider M B; Standop J; Ulrich A; Wittel U; Friess H; Andren-Sandberg A ; Pour P M

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journal of histochemistry and cytochemistry - official journal of the Histochemistry Society (United States) Oct 2001 , 49 (10) p1205-10, ISSN 0022-1554--Print Journal Code: 9815334

Contract/Grant No.: 5ROICA60479; CA; NCI; CA367127; CA; NCI; P50CA72712;
CA; NCI

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

One of the characteristics of pancreatic **cancer** is its tendency to invade neural tissue. We hypothesized that the affinity of **cancer** cells for nerve tissue is related to the presence of growth factors in neural tissue and their receptors in **cancer** cells. Sections of pancreatic **cancer** and normal pancreatic tissue were examined by immunohistochemistry for the expression of the neurotrophins NGF, BDNF, NT-3, **NT - 4**, and their receptors TrkA, **TrkB**, and TrkC, as well as the low-affinity receptor, p75NTR. TrkA expression was found in duct, islet, and **cancer** cells; **TrkB** was found in the alpha-cells of the islet only. The anti-pan-Trk antibody (TrkB3), which is presumed to recognize all three receptors, immunoreacted with duct and acinar cells in normal tissue and with **cancer** cells. The staining with TrkC was similar to that of TrkA. The low-affinity receptor p75NTR was expressed in the neural tissue and in scattered duct cells of the normal tissue only. Duct and acinar cells, as well as neural tissue and **cancer** cells, showed weak to strong immunoreactivity with NGF. NT-3 expression was noted in capillary endothelia and erythrocytes. **NT - 4** showed specific staining for ductule cells. The expression and distribution of neurotrophins and their receptors suggest their role in the potential of pancreatic **cancer** cells for neural invasion.

Expression of nerve growth factors in pancreatic neural tissue and pancreatic cancer .

... 2001 ,

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13/3,K,AB/3 (Item 3 from file: 155)

DIALOG(R)File 155:MEDLINE(R)

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13223917 PMID: 11359788

Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways.

Descamps S; Toillon R A; Adriaenssens E; Pawlowski V; Cool S M; Nurcombe V; Le Bourhis X; Boilly B; Peyrat J P; Hondermarck H

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Journal of biological chemistry (United States) May 25 2001 , 276 (21) p17864-70, ISSN 0021-9258--Print Journal Code: 2985121R

Publishing Model Print-Electronic

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

We show here that the neurotrophin nerve growth factor (NGF), which has been shown to be a mitogen for breast **cancer** cells, also stimulates cell survival through a distinct signaling pathway. Breast **cancer** cell lines (MCF-7, T47-D, BT-20, and MDA-MB-231) were found to express both types of NGF receptors: p140(trkA) and p75(NTR). The two other tyrosine kinase receptors for neurotrophins, **TrkB** and TrkC, were not expressed. The mitogenic effect of NGF on breast **cancer** cells required the tyrosine kinase activity of p140(trkA) as well as the mitogen-activated protein kinase (MAPK) cascade, but was independent of p75(NTR). In contrast, the anti-apoptotic effect of NGF (studied using the ceramide analogue C2) required p75(NTR) as well as the activation of the transcription factor NF-kB, but neither p140(trkA) nor MAPK was necessary. Other neurotrophins (BDNF, NT-3, **NT - 4 /5**) also induced cell survival, although not proliferation, emphasizing the importance of p75(NTR) in NGF-mediated survival. Both the pharmacological NF-kappaB inhibitor SN50, and cell transfection with IkBm, resulted in a diminution of NGF anti-apoptotic effect. These data show that two distinct signaling pathways are required for NGF activity and confirm the roles played by p75(NTR) and NF-kappaB in the activation of the survival pathway in breast **cancer** cells.

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Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways.

... 2001 ,

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... by p75(NTR) and NF-kappaB in the activation of the survival pathway in breast **cancer** cells.

...; Growth Factor--metabolism--ME; Receptor, trkA--metabolism--ME; Research Support, Non-U.S. Gov't; **Tumor** Cells, Cultured

13/3,K,AB/4 (Item 4 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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13118856 PMID: 11223544

Signal transduction pathways through TRK-A and TRK-B receptors in human neuroblastoma cells.

Sugimoto T; Kuroda H; Horii Y; Moritake H; Tanaka T; Hattori S

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Japanese journal of cancer research - Gann (Japan) Feb 2001 , 92 (2)
p152-60, ISSN 0910-5050--Print Journal Code: 8509412

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Main Citation Owner: NLM

Record type: MEDLINE; Completed

Little is known about the signal transduction pathways of TRK family receptors in neuroblastoma (NB) cells. In this study, an NB cell line, designated MP-N-TS, was established from an adrenal **tumor** taken from a 2-year-old boy. This cell line expressed both TRK-A and TRK-B receptors, which is rare in a single NB cell line. Therefore, the MP-N-TS cell line was used to determine whether the signal transduction through these constitutive receptors is functional. Three neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-4 / 5 (**NT - 4** / 5), induced tyrosine phosphorylation of panTRK, and BDNF and **NT - 4** / 5 induced tyrosine phosphorylation of TRK-B. Tyrosine phosphorylation of panTRK and / or TRK-B by the neurotrophins was inhibited in the presence of a tyrosine kinase inhibitor K252a. Tyrosine phosphorylation of Src homologous and collagen (Shc), extracellular signal-regulated kinase (ERK)-1 and ERK-2, and phospholipase C-gamma1 (PLC-gamma1) was increased by the three neurotrophins and the increase was inhibited in the presence of K252a. Activation of Ras, detected as the GTP-bound form of Ras, was induced by the three neurotrophins. The neurotrophins did not modulate the expressions of TRK-A or TRK-B mRNA, but they did induce the expression of c-fos mRNA. Exogenous NGF induced weak neurite outgrowth, whereas exogenous BDNF and **NT - 4** / 5 induced distinct

neurite outgrowth. Exogenous BDNF and **NT - 4** / 5 increased the number of viable cells, while NGF did not. Our results demonstrate that the signal transduction pathways through TRK-A and TRK-B in MP-N-TS cells are functional and similar, and the main downstream signaling pathways from the three neurotrophins are mitogen-activated protein kinase (MAPK) cascades through Shc, activated Ras, ERK-1 and ERK-2, and the transduction pathway through PLC-gamma1. Further, BDNF and **NT - 4** / 5 increased cell viability. The MP-N-TS cell line should be useful for clarifying the TRK-A and TRK-B signaling pathways responsible for the different prognoses in patients with NB.

... 2001 ,

... this study, an NB cell line, designated MP-N-TS, was established from an adrenal **tumor** taken from a 2-year-old boy. This cell line expressed both TRK-A and...

... Three neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-4 / 5 (**NT - 4** / 5), induced tyrosine phosphorylation of panTRK, and BDNF and **NT - 4** / 5 induced

tyrosine phosphorylation of TRK-B. Tyrosine phosphorylation of panTRK and / or TRK-B...

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Descriptors: *Neuroblastoma--metabolism--ME; *Receptor, trkA--metabolism--ME; *Receptor, trkB--metabolism--ME; *Signal Transduction...; Proteins p21(ras)--metabolism--ME; RNA, Messenger--biosynthesis--BI; Rats; Receptor, trkA--genetics--GE; Receptor, trkB--genetics--GE; Research Support, Non-U.S. Gov't; Tumor Cells, Cultured

Enzyme No.: EC 2.7.1.112 (Receptor, trkA); EC 2.7.1.112 (Receptor, trkB); EC 3.6.5.2 (HRAS protein, human); EC 3.6.5.2 (Proto-Oncogene

...Chemical Name: Growth Factors; Proto-Oncogene Proteins c-fos; RNA, Messenger; Phosphotyrosine; K 252; Receptor, trkA; Receptor, trkB; HRAS protein, human; Proto-Oncogene Proteins p21(ras)

13/3,K,AB/5 (Item 5 from file: 155)

DIALOG(R)File 155:MEDLINE(R)

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12102439 PMID: 10545017

Role of neurotrophins and neurotrophins receptors in the in vitro invasion and heparanase production of human prostate cancer cells.

Walch E T; Marchetti D

Department of Cancer Biology, The University of Texas M.D. Anderson Cancer Center, Houston, USA.

Clinical & experimental metastasis (NETHERLANDS) Jun 1999, 17 (4)

p307-14, ISSN 0262-0898--Print Journal Code: 8409970

Contract/Grant No.: R29-CA64178; CA; NCI

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

The role of the neurotrophins (NTs) and their corresponding receptors (NTRs) TrkA, TrkB, TrkC, and p75NTR in neoplasia has received relatively little attention. However, because malignant cell migration within the prostate occurs predominantly by direct extension around prostatic nerves, the presence and possible upregulation of NTs from autocrine/paracrine sources and NTR expression within prostate epithelial tumor cells may be important in metastasis. We have been addressing their expression and interactions in human prostate cancer cell lines (LNCaP, PC-3, and DU145) and their role in prostate cancer invasion. In this study, we demonstrated that nerve growth factor (NGF), the prototypic NT, and NT - 4 / 5 increased in vitro invasion through a reconstituted basement membrane and induced time- and dose-dependent expression of heparanase, a heparan sulfate-specific endo-beta-D-glucuronidase, an important molecular determinant of tumor metastasis. The NT effects were most marked in the DU 145 brain-metastatic cells and were detected at NT concentrations sufficient to fully saturate both low- and high-affinity NTRs.

Additionally, we characterized the molecular expression of NT high-affinity (Trk) and low-affinity (p75NTR) receptors in these cell lines by reverse transcription-polymerase chain reaction. These lines had negligible trkA and trkC expression, although **trkB** was expressed in the three prostatic

tumor cell lines examined. The brain-metastatic DU 145 cells were also positive for p75NTR. Our data showed that the NTs and NTRs are important in metastasis and that their expression coincides with transformation to a **malignant** phenotype capable of invasion along the perineural space and extracapsular metastasis to distant sites. These findings set the stage for more research into this area as related to prostate **cancer** evolution and may improve therapy for prostate **cancer** metastasis.

...neurotrophins and neurotrophins receptors in the in vitro invasion and heparanase production of human prostate cancer cells.

... 1999 ,

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...and NTRs are important in metastasis and that their expression coincides with transformation to a malignant phenotype capable of invasion along the perineural space and extracapsular metastasis to distant sites. These findings set the stage for more research into this area as related to prostate cancer evolution and may improve therapy for prostate cancer metastasis.

...; t; Research Support, U.S. Gov't, P.H.S.; Reverse Transcriptase Polymerase Chain Reaction; Tumor Cells, Cultured

13/3,K,AB/6 (Item 6 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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11576307 PMID: 9417837

Truncated trkB receptors on nonneuronal cells inhibit BDNF-induced neurite outgrowth in vitro.

Fryer R H; Kaplan D R; Kromer L F

Department of Cell Biology, Georgetown University Medical Center,

Washington, DC 20007, USA.

Experimental neurology (UNITED STATES) Dec 1997 , 148 (2) p616-27,
ISSN 0014-4886--Print Journal Code: 0370712

Contract/Grant No.: NS31445; NS; NINDS

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

The function of truncated **trkB** receptors during nervous system plasticity and regeneration is currently unknown. The extensive nonneuronal localization of truncated **trkB** -T1 receptors, coupled with their up-regulation by CNS glial cells in response to injury, has led to the speculation that these receptors may sequester BDNF and **NT - 4 / 5** to reduce their local availability and, thus, limit axonal sprouting. Conversely, **trkB** -T1 receptors could bind and present neurotrophins to injured axons and facilitate their regeneration in a manner analogous to that proposed for p75(NTR) receptors on Schwann cells. To address this issue, we used an in vitro coculture paradigm in which wild-type 3T3 NIH fibroblasts or two different 3T3 cell clones stably expressing **trkB** -T1 receptors served as monolayer substrates upon which to evaluate the effect of **trkB** -T1 receptors on nonneuronal cells to influence neurotrophin (NGF, BDNF, **NT-3**, and **NT - 4 / 5**)-induced neurite outgrowth from retinoic acid (RA)-treated SY5Y neuroblastoma cells. In these experiments, BDNF and **NT - 4 / 5** produce a strong phosphorylation of **trk** receptors on the RA-SY5Y cells and induce differentiation of the SY5Y cells (as measured by the development of neurofilament-positive neuritic processes). This ability of the **trkB** ligands to stimulate neurite outgrowth is dose dependent since increasing concentrations of BDNF (5, 25, and 100 ng/ml) result in an increased percentage of SY5Y cells developing neurites and in progressively longer neurites from SY5Y cells on the control 3T3 monolayers. In these experiments, BDNF and **NT - 4 / 5** induce the strongest neurite outgrowth, followed by **NT-3** and then NGF. When **trkB** -T1 receptors are present on the 3T3 cell substratum both BDNF- and **NT - 4 / 5**-induced neurite extension from the SY5Y cells are strongly inhibited. In contrast, NGF-induced neurite growth is unaffected and **NT-3**-associated growth is somewhat reduced. These results suggest that the inhibitory effect of the **trkB** -T1 receptors on the nonneuronal cell substrates is selective for neurite outgrowth that is mediated via the **trkB** -kinase receptors on the neuroblastoma cells. This ability of **trkB** -T1 receptors on the nonneuronal substratum to inhibit BDNF-induced neurite outgrowth can be overcome by the addition of high concentrations of BDNF (1 microg/ml). Binding assays using ¹²⁵I-BDNF suggest that this inhibitory effect could be mediated via binding and internalization of BDNF by the **trkB** -T1 receptors on the 3T3 cells. These results provide strong support for the hypothesis that the up-regulation of **trkB** -T1 receptors on astrocytes following CNS lesions enhances the sequestration of the **trkB** ligands, BDNF and **NT - 4 / 5**, at the site of reactive gliosis and, thus, contributes to the inhibition of CNS axonal regeneration from neurons expressing **trkB** -kinase receptors by removing their ligands from the extracellular environment. Copyright 1997 Academic Press.

Truncated **trkB receptors on nonneuronal cells inhibit BDNF-induced neurite outgrowth in vitro.**

... 1997 ,

The function of truncated **trkB** receptors during nervous system plasticity and regeneration is currently unknown. The extensive nonneuronal

localization of truncated **trkB** -T1 receptors, coupled with their up-regulation by CNS glial cells in response to injury, has led to the speculation that these receptors may sequester BDNF and **NT - 4 / 5** to reduce their local availability and, thus, limit axonal sprouting. Conversely, **trkB** -T1 receptors could bind and present neurotrophins to injured axons and facilitate their regeneration in...

... in which wild-type 3T3 NIH fibroblasts or two different 3T3 cell clones stably expressing **trkB** -T1 receptors served as monolayer substrates upon which to evaluate the effect of **trkB** -T1 receptors on nonneuronal cells to influence neurotrophin (NGF, BDNF, NT-3, and **NT - 4 / 5**)-induced neurite outgrowth from retinoic acid (RA)-treated SY5Y neuroblastoma cells. In these experiments, BDNF and **NT - 4 / 5** produce a strong phosphorylation of **trk** receptors on the RA-SY5Y cells and induce...

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...; Research Support, U.S. Gov't, P.H.S.; Sequence Deletion; Transfection; Tretinoin--pharmacology--PD; **Tumor** Cells, Cultured

13/3,K,AB/7 (Item 7 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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11565614 PMID: 9406845

Molecular characterization of neurotrophin expression and the corresponding tropomyosin receptor kinases (trks) in epithelial and stromal cells of the human prostate.

Dalal R; Djakiew D

Department of Cell Biology, Georgetown University Medical Center, Washington, DC 20007, USA.

Molecular and cellular endocrinology (IRELAND) Oct 31 1997 , 134 (1)

p15-22, ISSN 0303-7207--Print Journal Code: 7500844
Contract/Grant No.: K04-DK-02233; DK; NIDDK; TW02137; TW; FIC
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

The prostate is one of the most abundant sources of nerve growth factor (NGF) outside of the nervous system. NGF is a member of the neurotrophin family of growth factors which in mammals also includes brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT - 4 /5). These neurotrophins can bind with high affinity to a family of tropomyosin receptor kinases (trks). These receptors are trkA, which binds NGF; **trkB** , which binds both BDNF and NT - 4 /5; and trkC, which binds NT-3. In order to characterize the molecular expression of the neurotrophins and their corresponding trk receptors in the prostate we performed Northern blot analysis for the neurotrophins and reverse transcription-polymerase chain reaction (RT-PCR) coupled with Southern blot analysis for the trk family of receptors on smooth muscle stromal cells from the prostate, the androgen responsive LNCaP prostate **tumor** cell line and the androgen refractory TSU-pr1 prostate **tumor** cell line. The results show that smooth muscle stromal cells expressed NGF, BDNF and trkC, whereas both epithelial cell lines expressed trkA, **trkB** and trkC to various degrees. NT-3 was not detected in either the smooth muscle stromal cells or in both epithelial cell lines. This suggests that the stromal cell derived NGF and BDNF may interact via paracrine mechanisms with trkA and **trkB** receptors, respectively, on the adjacent epithelial cells. Interestingly, the androgen responsive LNCaP cell line did not express any of the neurotrophins, whereas the androgen refractory TSU-pr1 cell line expressed NGF, BDNF and NT - 4 /5. This suggests that the autocrine expression of NGF, BDNF and NT - 4 /5 is up-regulated in prostate epithelial cells following their transformation to an androgen refractory pathology. Hence, the **malignant** transformation of prostate epithelial **tumor** cells may facilitate their escape from a paracrine dependence on stromal cell derived neurotrophins by the acquisition of the autocrine expression of neurotrophins. Since the pathology of **malignant** cell migration within the prostate is predominantly by direct extension around prostatic nerves the upregulation of autocrine neurotrophin expression within prostate epithelial **tumor** cells may be concomitant with transformation to a **malignant** phenotype capable of invasion along the perineural space and extracapsular metastasis to distant sites of **tumor** formation.

... 1997 ,

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...; U.S. Gov't, P.H.S.; Stromal Cells--chemistry--CH; Stromal Cells --enzymology--EN; **Tumor** Cells, Cultured

13/3,K,AB/8 (Item 8 from file: 155)

DIALOG(R)File 155:MEDLINE(R)

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11210423 PMID: 8978713

K-252b potentiation of neurotrophin-3 is trkA specific in cells lacking p75NTR.

Maroney A C; Sanders C; Neff N T; Dionne C A

Cephalon Inc., West Chester, Pennsylvania 19380, USA.

Journal of neurochemistry (UNITED STATES) Jan 1997 , 68 (1) p88-94,

ISSN 0022-3042--Print Journal Code: 2985190R

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

K-252b potentiates the neurotrophic effects of neurotrophin-3 (NT-3) in primary cultures of rat central cholinergic and peripheral sensory neurons and in a rat pheochromocytoma PC12 cell line. The ligand and receptor specificity, and role of the low-affinity neurotrophin receptor (p75NTR) in the potentiation response induced by K-252b, are unknown. To address the issues of ligand and receptor specificity of K-252b potentiation, we have examined neurotrophin-induced DNA synthesis ([3H]-thymidine incorporation) in NIH3T3 cells expressing trkA, **trkB**, or trkC. Neither NT-3 nor K-252b alone could stimulate mitogenic activity in the trkA-overexpressing clone. However, coaddition of K-252b (EC50 of approximately 2 nM) with 10-100 ng/ml NT-3 led to incorporation of [3H]thymidine in trkA expressing cells to a level induced by optimal concentrations of nerve growth factor (NGF). The K-252b- and NT-3-induced [3H]thymidine incorporation correlated with an increase in the tyrosine autophosphorylation of the trkA receptor as well as tyrosine phosphorylation of trk-associated phospholipase C-gamma 1 and SH2-containing proteins. K-252b did not potentiate submaximal doses of NGF, or maximal doses of brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5 (NT - 4 / 5) in trkA-expressing cells. Furthermore, K-252b did not potentiate DNA synthesis by submaximal doses of BDNF, NT - 4 /5, or NT-3 in **trkB** - or trkC-expressing NIH3T3 cells, suggesting that the potentiation profile for K-252b was specific for NT-3 in

trkA-expressing cells. We found no expression of p75NTR in the trk-expressing NIH3T3 cells. This is the first demonstration that K-252b potentiates a trkA-mediated biological nonneuronal response by NT-3 that occurs independent of p75NTR and appears to be both ligand and receptor specific.

... 1997 ,

... we have examined neurotrophin-induced DNA synthesis ([3H]-thymidine incorporation) in NIH3T3 cells expressing trkA, **trkB** , or trkC. Neither NT-3 nor K-252b alone could stimulate mitogenic activity in the...

... of NGF, or maximal doses of brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5 (**NT - 4 / 5**) in trkA-expressing cells. Furthermore, K-252b did not potentiate DNA synthesis by submaximal doses of BDNF, **NT - 4 / 5**, or NT-3 in **trkB** - or trkC-expressing NIH3T3 cells, suggesting that the potentiation profile for K-252b was specific...

...; Phosphorylation; Rats; Receptor, trkA--genetics--GE; Receptors, Nerve Growth Factor--physiology--PH; Substrate Specificity; Transfection; **Tumor** Cells, Cultured

13/3,K,AB/9 (Item 9 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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10528403 PMID: 7623136

Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity.

Zheng J L; Stewart R R; Gao W Q

Department of Neuroscience, Genentech, Inc., South San Francisco, California 94080, USA.

Journal of neuroscience - the official journal of the Society for Neuroscience (UNITED STATES) Jul 1995 , 15 (7 Pt 2) p5079-87, ISSN 0270-6474--Print Journal Code: 8102140

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Destruction of spiral ganglion neurons (SGNs) induced by injury and toxins is one of the major causes for hearing loss. Here we report that neurotrophin-4/5 (**NT - 4 / 5**), a member of the nerve growth factor family, promoted survival of postnatal rat SGNs up to threefold in dissociated cell cultures. The survival-promoting potency of **NT - 4 / 5** was equivalent to that of BDNF and stronger than that of NT-3. In contrast, NGF showed no detectable effects. Immunohistochemistry, with **TrkB** and TrkA antisera, revealed that these neurons produced **TrkB** protein, the functional receptor for **NT - 4 / 5** and BDNF, but not TrkA protein, the high-affinity receptor for NGF. The survival-promoting activity of **NT - 4 / 5** was completely inhibited by **TrkB** -IgG fusion protein. These results suggest that **NT - 4 / 5** is a specific survival factor for SGNs. In addition, **NT - 4 / 5** protected the SGNs from neurotoxic effects of the anti- **cancer** drug, cisplatin. Thus, **NT - 4 / 5** may have therapeutic value in preventing hearing impairment caused by damage to primary auditory afferent neurons.

... 1995 ,

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13/3,K,AB/10 (Item 10 from file: 155)
DIALOG(R) File 155:MEDLINE(R)
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09721389 PMID: 8394722

Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. Eukaryotic Signal Transduction Group.

Kaplan D R; Matsumoto K; Lucarelli E; Thiele C J
ABL-Basic Research Program, National Cancer Institute, Frederick Cancer Research and Development Center, Maryland 21702.

Neuron (UNITED STATES) Aug 1993 , 11 (2) p321-31, ISSN 0896-6273--
Print Journal Code: 8809320

Contract/Grant No.: N01-CO-74101; CO; NCI

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Retinoic acid (RA) induces the neuronal differentiation of many human neuroblastoma cell lines. In this study, we show that RA treatment of neuroblastoma cells induces the expression of **TrkB**, the receptor for the neurotrophins BDNF, NT-3, and NT - 4 /5. BDNF addition to RA-treated SH-SY5Y neuroblastoma cells stimulated the tyrosine phosphorylation of **TrkB** and neuronal differentiation. RA treatment of KCNR neuroblastoma cells, which constitutively express BDNF mRNA, resulted in the expression of **TrkB** and differentiation in the absence of added BDNF. Finally, in 15N neuroblastoma cells, which express BDNF mRNA but do not differentiate in response to RA, RA induced only a truncated form of **TrkB**. 15N cells transfected with full-length **TrkB** differentiated in the absence of RA. These results indicate that RA induces the neuronal differentiation of neuroblastoma cells by modulating the expression of neurotrophin receptors.

Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. Eukaryotic...

... 1993 ,

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... but do not differentiate in response to RA, RA induced only a truncated form of **TrkB**. 15N cells transfected with full-length **TrkB** differentiated in the absence of RA. These results indicate that RA induces the neuronal differentiation...

...; **trkA**; Receptors, Cell Surface--metabolism--ME; Research Support, U.S. Gov't, P.H.S.; **Tumor** Cells, Cultured--drug effects--DE; **Tumor** Cells, Cultured--pathology--PA; Tyrosine--metabolism--ME

13/3,K,AB/11 (Item 11 from file: 155)

DIALOG(R)File 155:MEDLINE(R)

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09232890 PMID: 1375038

The trkB tyrosine protein kinase is a receptor for neurotrophin-4.

Klein R; Lamballe F; Bryant S; Barbacid M

Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000.

Neuron (UNITED STATES) May 1992, 8 (5) p947-56, ISSN 0896-6273--
Print Journal Code: 8809320

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Neurotrophin-4 is a novel member of the nerve growth factor family of neurotrophins recently isolated from *Xenopus* and viper DNA. We now report that the *Xenopus* NT - 4 protein (XNT-4) can mediate some of its biological properties through gp145trkB, a murine tyrosine protein kinase previously identified as a primary receptor for the related brain-derived neurotrophic factor (BDNF). XNT-4 displaces 125I-labeled BDNF from binding to cells expressing gp145trkB receptors, induces their rapid phosphorylation on tyrosine residues, and causes the morphologic transformation of NIH 3T3 cells when coexpressed with gp145trkB. Moreover, XNT-4 induces the differentiation of PC12 cells into sympathetic-like neurons only if they ectopically express gp145trkB receptors. None of these biochemical or biological effects could be observed when XNT-4 was added to cells expressing the related receptors. Replacement of one of the extracellular cysteines (Cys-345) of gp145trkB by a serine residue prevents its activation by XNT-4 but not by BDNF. Therefore, XNT-4 and BDNF may interact with at least partially distinct domains within the gp145trkB receptor.

The trkB tyrosine protein kinase is a receptor for neurotrophin-4.

... 1992 ,

... of neurotrophins recently isolated from *Xenopus* and viper DNA. We now report that the *Xenopus* NT - 4 protein (XNT-4) can mediate some of its biological properties through gp145trkB, a murine tyrosine...

...; Proteins--metabolism--ME; Neurons--cytology--CY; Pheochromocytoma; Phosphorylation; Phosphotyrosine; Protein-Tyrosine Kinase--genetics--GE; Transfection; **Tumor** Cells, Cultured; Tyrosine--analogs and derivatives --AA; Tyrosine--metabolism--ME; *Xenopus*

13/3,K,AB/12 (Item 1 from file: 55)

DIALOG(R)File 55:Biosis Previews(R)

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0012506542 BIOSIS NO.: 200000224855

**Neurotrophin and neurotrophin-receptor expression in primitive
neuroectodermal brain tumors**

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**Neurotrophin and neurotrophin-receptor expression in primitive
neuroectodermal brain tumors**

2000

DESCRIPTORS:

...MAJOR CONCEPTS: Tumor Biology

DISEASES: primitive neuroectodermal brain tumor --

CHEMICALS & BIOCHEMICALS: ... NT - 4 --...

... TrkB --

13/3,K,AB/13 (Item 2 from file: 55)

DIALOG(R) File 55:Biosis Previews(R)

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**Expression of the naturally occurring truncated trkB neurotrophin
receptor induces outgrowth of filopodia and processes in neuroblastoma
cells**

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DOCUMENT TYPE: Article

RECORD TYPE: Abstract

LANGUAGE: English

ABSTRACT: We have investigated the effects of the truncated **trkB** receptor
isoform T1 (**trkB** .T1) by transient transfection into mouse N2a
neuroblastoma cells. We observed that expression of **trkB** .T1 leads to a
striking change in cell morphology characterized by outgrowth of
filopodia and processes. A similar morphological response was also
observed in SH-SY5Y human neuroblastoma cells and NIH3T3 fibroblasts
transfected with **trkB** .T1. N2a cells lack endogenous expression of **trkB**
isoforms, but express barely detectable amounts of its ligands,
brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (**NT - 4**).

The morphological change was ligand-independent, since addition of exogenous BDNF or NT - 4 or blockade of endogenous **trkB** ligands did not influence this response. Filopodia and process outgrowth was significantly suppressed when full-length **trkB** .TK+ was cotransfected together with **trkB** .T1 and this inhibitory effect was blocked by tyrosine kinase inhibitor K252a. Transfection of **trkB** .T1 deletion mutants showed that the morphological response is dependent on the extracellular, but not the intracellular domain of the receptor. Our results suggest a novel ligand-independent role for truncated **trkB** in the regulation of cellular morphology.

Expression of the naturally occurring truncated trkB neurotrophin receptor induces outgrowth of filopodia and processes in neuroblastoma cells
1999

ABSTRACT: We have investigated the effects of the truncated **trkB** receptor isoform T1 (**trkB** .T1) by transient transfection into mouse N2a neuroblastoma cells. We observed that expression of **trkB** .T1 leads to a striking change in cell morphology characterized by outgrowth of filopodia and...

...response was also observed in SH-SY5Y human neuroblastoma cells and NIH3T3 fibroblasts transfected with **trkB** .T1. N2a cells lack endogenous expression of **trkB** isoforms, but express barely detectable amounts of its ligands, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT - 4). The morphological change was ligand-independent, since addition of exogenous BDNF or NT - 4 or blockade of endogenous **trkB** ligands did not influence this response. Filopodia and process outgrowth was significantly suppressed when full-length **trkB** .TK+ was cotransfected together with **trkB** .T1 and this inhibitory effect was blocked by tyrosine kinase inhibitor K252a. Transfection of **trkB** .T1 deletion mutants showed that the morphological response is dependent on the extracellular, but not...

...intracellular domain of the receptor. Our results suggest a novel ligand-independent role for truncated **trkB** in the regulation of cellular morphology.

DESCRIPTORS:

MAJOR CONCEPTS: Tumor Biology

CHEMICALS & BIOCHEMICALS: ... **trkB** receptor isoform T1 { **trkB** .T1...

... **trkB** --

13/3,K,AB/14 (Item 1 from file: 340)

DIALOG(R) File 340:CLAIMS(R)/US Patent

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IFI Publication Control No: 2002-0146416 IFI Chemical Acc No: 2002-0037518

Document Type: C

HUMAN TRK RECEPTORS AND NEUROTROPHIC FACTOR INHIBITORS; CONTACTING NEUROTROPHIN RECEPTOR WITH AN ANTIBODY SPECIFIC FOR NEUROTROPHIN RECEPTOR, FOR INHIBITING OR ENHANCING A BIOLOGICAL ACTIVITY MEDIATED BY A NEUROTROPHIN RECEPTOR

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Publication (No,Kind,Date), Applic (No,Date):
US 20020146416 A1 20021010 US 2001966147 20010927
Priority Applic(No,Date): US 2001966147 20010927; US 94286846
19940805; US 95446172 19950519; US 94215139 19940318

Abstract: The invention concerns human **trkB** and **trkC** receptors and their functional derivatives. The invention further concerns immunoadhesins comprising **trk** receptor sequences fused to immunoglobulin sequences.
Publication (No,Kind,Date), Applic (No,Date):
... 20021010

Abstract: The invention concerns human **trkB** and **trkC** receptors and their functional derivatives. The invention further concerns immunoadhesins comprising **trk** receptor...

Exemplary Claim:

...receptor, wherein said neurotrophin receptor is selected from the group consisting of human **TrkA**, human **TrkB** and human **TrkC**.

Non-exemplary Claims:

- ...9. The method of claim 1 wherein said human **TrkB** comprises the sequence of SEQ ID NO: 2 or SEQ ID NO: 4...
- ...12. The method of claim 1 wherein said biological activity is **tumor** development, and said **tumor** development is inhibited...
- ...13. The method of claim 1 wherein said biological activity is **cancer** development, and said **cancer** development is inhibited...
- ...further wherein said neurotrophin receptor is selected from the group consisting of human **TrkA**, human **TrkB** and human **TrkC**...
- ...from the group consisting of inflammatory pain, pancreas disorders, kidney disorders, lung disorders, cardiovascular disorders, **tumors**, **cancers**, aberrant neuron sprouting, neurodegenerative diseases and psychiatric disorders...
- ...further wherein said neurotrophin receptor is selected from the group consisting of human **TrkA**, human **TrkB** and human **TrkC**...
- ...further wherein said neurotrophin receptor is selected from the group consisting of human **TrkA**, human **TrkB** and human **TrkC**...
- ...neurotrophin is selected from the group consisting of NGF, BDNF/Neurotrophin-2, NT-3, and **NT - 4** /5...
- ...antibody, wherein said neurotrophin receptor is selected from the group consisting of human **TrkA**, human **TrkB** and human **TrkC**...
- ...neurotrophin, wherein said neurotrophin receptor is selected from the group consisting of human **TrkA**, human **TrkB** and human **TrkC**...

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(A1) TREATMENT OF BALANCE IMPAIRMENTS; INVOLVING NEURONAL DAMAGE, LOSS, OR DEGENERATION, SUCH AS OF VESTIBULAR GANGLION NEURONS, BY ADMINISTRATION OF A TYROSINE KINASE-CONTAINING RECEPTOR (TRK) TRKB OR TRKC AGONIST SUCH AS A NEUROTROPHIN; TREATING OTOTOXICITY

(B2) TREATMENT OF BALANCE IMPAIRMENTS; INVOLVING NEURONAL DAMAGE, LOSS, OR DEGENERATION, SUCH AS OF VESTIBULAR GANGLION NEURONS, BY ADMINISTRATION OF A TYROSINE KINASE-CONTAINING RECEPTOR (TRK) TRKB OR TRKC AGONIST SUCH AS A NEUROTROPHIN; TREATING OTOTOXICITY

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US 20020169124 A1 20021114 US 200296762 20020312

US 6974798 B2 20051213 US 200296762 20020312

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Notes: INDEXED FROM APPLICATION Subject to any Disclaimer, the term of this patent is extended or adjusted under 35 USC 154(b) by 306 days.

Prior Publication(No,Date),Applic(No,Date):US 20020169124 A1 20021114

Continuation Pub(No),Applic(No,Date): (US 20020169124 A1) Compositions and methods are provided for prophylactic or therapeutic treatment of balance impairments involving neuronal damage, loss, or degeneration, preferably of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 / 5** (US 6974798 B2) Compositions and methods are provided for prophylactic or therapeutic treatment of balance impairments involving neuronal damage, loss, or degeneration, preferably of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 / 5**.

Priority Applic(No,Date): US 200296762 20020312; US 95581662

19951229; US 2000664295 20000918

Abstract: (US 20020169124 A1)

Compositions and methods are provided for prophylactic or therapeutic treatment of balance impairments involving neuronal damage, loss, or degeneration, preferably of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 / 5**

Abstract: (US 6974798 B2)

Compositions and methods are provided for prophylactic or therapeutic treatment of balance impairments involving neuronal damage, loss, or degeneration, preferably of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 / 5**.

...SUCH AS OF VESTIBULAR GANGLION NEURONS, BY ADMINISTRATION OF A TYROSINE KINASE-CONTAINING RECEPTOR (TRK) TRKB OR TRKC AGONIST SUCH AS A NEUROTROPHIN; TREATING OTOTOXICITY...

...SUCH AS OF VESTIBULAR GANGLION NEURONS, BY ADMINISTRATION OF A TYROSINE

KINASE-CONTAINING RECEPTOR (TRK) TRKB OR TRKC AGONIST SUCH AS A NEUROTROPHIN; TREATING OTOTOXICITY

Publication (No,Kind,Date), Applic (No,Date):

... 20021114

...Prior Publication(No,Date),Applic(No,Date): 20021114

...Continuation Pub(No),Applic(No,Date): of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 /5**...

...of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 /5**.

...Cont.-in-part Pub(No),Applic(No,Date): administering to a mammal in need of such treatment a therapeutically effective amount of a **trkB** or **trkC** agonist...

...A pharmaceutical composition, comprising a pharmaceutical agent capable of ototoxin-induced balance impairment and a **trkB** or **trkC** agonist in an amount therapeutically effective for treating ototoxicity caused by the pharmaceutical agent, wherein said agonist is (a) a **NT - 4 /5** (SEQ ID NO:1) variant comprising one to five amino acid alterations selected from

...single amino acid or deletion of the indicated span of residues, inclusive; or (b) a **NT - 4 /5** (SEQ ID NO:1) fragment selected from the group consisting of **NT - 4 /5**(R83-Q94), **NT - 4 /5**(G1-C61), **NT - 4 /5**(G1-C17), **NT - 4 /5**(C17-C61), **NT - 4 /5**(C17-C78), **NT - 4 /5**(C17-C90), **NT - 4 /5**(C17C119), **NT - 4 /5**(C17-C121), **NT - 4 /5**(R11-R27), **NT - 4 /5**(R11-R34), **NT - 4 /5**(R34-R53), **NT - 4 /5**(C61-C78), **NT - 4 /5**(R53-C61), **NT - 4 /5**(C61-C119), **NT - 4 /5**(C61-C78), **NT - 4 /5**(C78-C119), **NT - 4 /5**(C61-C90), **NT - 4 /5**(R60C78), **NT - 4 /5**(K62-C119), **NT - 4 /5**(K62-K91), **NT - 4 /5**(R79-R98), **NT - 4 /5**(R83-R93), **NT - 4 /5**(T101-R111), **NT - 4 /5**(G1-C121)VLTVKRVRR (SEQ ID NO:4), **NT - 4 /5**(V40-C121)VLTVKRVRR (SEQ ID NO:5), **NT - 4 /5**(V40-C121)SLTIKRIRA (SEQ ID NO:6), **NT - 4 /5**(V40-C121)TLSRKAGRRA (SEQ ID NO:7), **NT - 4 /5**(V40-C121)DDDSPIARRGEISVCDSDWVSAPDKDTAVDIKGDDVMVLKKVGINHSV (SEQ ID NO:8), and **NT - 4 /5**(V40-C121).

...Division Pub(No),Applic(No,Date): The method of claim 2, wherein the agonist is selected from the group consisting of **NT - 4 /5**, BDNF or **NT-3**

...4. The method of claim 3, wherein the agonist is **NT - 4 /5**...

...12. The method of claim 7, wherein the **trkB** or **trkC** agonist is administered prior to administration of an ototoxin...

...13. The method of claim 1, wherein the **trkB** or **trkC** agonist is administered with an agent that promotes hair cell growth or regeneration

...The method of claim 1, which further comprises administering an effective amount of a second **trkB** or **trkC** agonist...

...17. A method of assaying for a **trkB** or **trkC** agonist that provides vestibular ganglion neuron protection or survival from an ototoxin, comprising, culturing a utricle explant, administering a **trkB** or **trkC** agonist to the culture, administering an ototoxin to the culture, and determining the amount of protection or survival compared to a control culture to which the **trkB** or **trkC** agonist was not administered...

...A pharmaceutical composition, comprising a pharmaceutical agent capable of ototoxin-induced balance impairment and a **trkB** or **trkC** agonist in an amount therapeutically effective for treating ototoxicity caused by the pharmaceutical...

...administration of an aminoglycoside antibiotic, the improvement comprising administering a therapeutically effective amount of a **trkB** or **trkC** agonist to the patient in need of such treatment to reduce or prevent ...

...22. An improved method for treatment of **cancer** in a mammal by administration of a chemotherapeutic compound, the improvement comprises administering a therapeutically effective amount of a **trkB** or **trkC** agonist to the patient in need of such treatment to reduce or prevent...
...causing neuronal damage, loss, or degeneration, comprising administering to the neuron an effective amount of **trkB** or **trkC** agonist ...

...administering to a mammal in need of such treatment a therapeutically effective amount of a **trkB** or **trkC** agonist wherein said agonist is (a) a **NT - 4 / 5** (SEQ ID NO:1) variant comprising one to five amino acid alterations selected from...

...single amino acid or deletion of the indicated span of residues, inclusive; or (b) a **NT - 4 / 5** (SEQ ID NO:1) fragment selected from the group consisting of **NT - 4 / 5**(R83-Q94), **NT - 4 / 5**(G1-C61), **NT - 4 / 5**(G1-C17), **NT - 4 / 5**(C17-C61), **NT - 4 / 5**(C17-C78), **NT - 4 / 5**(C17-C90), **NT - 4 / 5**(C17-C119), **NT - 4 / 5**(C17-C121), **NT - 4 / 5**(R11-R27), **NT - 4 / 5**(R11-R34), **NT - 4 / 5**(R34-R53), **NT - 4 / 5**(C61-C78), **NT - 4 / 5**(R53-C61), **NT - 4 / 5**(C61-C119), **NT - 4 / 5**(C61-C78), **NT - 4 / 5**(C78-C119), **NT - 4 / 5**(C61-C90), **NT - 4 / 5**(R60-C78), **NT - 4 / 5**(K62-C119), **NT - 4 / 5**(K62-K91), **NT - 4 / 5**(R79-R98), **NT - 4 / 5**(R83-R93), **NT - 4 / 5**(T101-R111), **NT - 4 / 5**(G1-C121)VLTVKRVRR (SEQ ID NO:4), **NT - 4 / 5**(V40-C121)VLTVKRVRR (SEQ ID NO:5), **NT - 4 / 5**(V40-C121)SLTIKRIRA (SEQ ID NO:6), **NT - 4 / 5**(V40-C121)TLSRKAGRRA (SEQ ID NO:7), **DDDSPIARRGEISVCDSVSDWVSAPDKDTAVDIKGDDVMVLKKVGINHSV** (SEQ ID NO:8), and **NT - 4 / 5**(V40-C121)...

...12. The method of claim 7, wherein the **trkB** or **trkC** agonist is administered prior to administration of an ototoxin...

...13. The method of claim 4, wherein the **trkB** or **trkC** agonist is administered with an agent that promotes hair cell growth or regeneration ...

...The method of claim 4, which further comprises administering an effective amount of a second **trkB** or **trkC** agonist...

...administration of an aminoglycoside antibiotic, the improvement comprising administering a therapeutically effective amount of a **trkB** or **trkC** agonist to the mammal in need of such treatment to reduce ototoxin-induced balance impairment associated with the antibiotic, wherein said agonist is (a) a **NT - 4 / 5** (SEQ ID NO:1) variant comprising one to five amino acid alterations selected from...

...single amino acid or deletion of the indicated span of residues,

inclusive; or (b) a NT - 4 / 5 (SEQ ID NO:1) fragment selected from the group consisting of NT - 4 / 5(R83-Q94), NT - 4 / 5(G1-C61), NT - 4 / 5(G1-C17), NT - 4 / 5(C17-C61), NT - 4 / 5(C17-C78), NT - 4 / 5(C17-C90), NT - 4 / 5(C17-C119), NT - 4 / 5(C17-C121), NT - 4 / 5(R11-R27), NT - 4 / 5(R11-R34), NT - 4 / 5(R34-R53), NT - 4 / 5(C61-C78), NT - 4 / 5(R53-C61), NT - 4 / 5(C61-C119), NT - 4 / 5(C61-C78), NT - 4 / 5(C78-C119), NT - 4 / 5(C61-C90), NT - 4 / 5(R60-C78), NT - 4 / 5(K62-C119), NT - 4 / 5(K62-K91), NT - 4 / 5(R79-R98), NT - 4 / 5(R83-R93), NT - 4 / 5(T101-R111), NT - 4 / 5(G1-C121) VLTVKRVRR (SEQ ID NO:4), NT - 4 / 5(V40-C121) VLTVKRVRR (SEQ ID NO:5), NT - 4 / 5(V40-C121) SLTIKRIRA (SEQ ID NO:6), NT - 4 / 5(V40-C121) TLSRKAGRRA (SEQ ID NO:7), DDDSPIARRGEISVCDSVSDWVSAPDKDTAVDIKGDDVMVLKKVGINHSV (SEQ ID NO:8), and NT - 4 / 5(V40-C121)...

...18. An improved method for the treatment of **cancer** in a mammal by administration of a chemotherapeutic compound, the improvement comprising administering a therapeutically effective amount of a **trkB** or **trkC** agonist to the mammal in need of such treatment to reduce ototoxin-induced balance impairment associated with the chemotherapeutic drug, wherein said agonist is (a) a NT - 4 / 5 (SEQ ID NO:1) variant comprising one to five amino acid alterations selected from...

...single amino acid or deletion of the indicated span of residues, inclusive; or (b) a NT - 4 / 5 (SEQ ID NO:1) fragment selected from the group consisting of NT - 4 / 5(R83-Q94), NT - 4 / 5(G1-C61), NT - 4 / 5(G1-C17), NT - 4 / 5(C17-C61), NT - 4 / 5(C17-C78), NT - 4 / 5(C17-C90), NT - 4 / 5(C17-C119), NT - 4 / 5(C17-C121), NT - 4 / 5(R11-R27), NT - 4 / 5(R11-R34), NT - 4 / 5(R34-R53), NT - 4 / 5(C61-C78), NT - 4 / 5(R53-C61), NT - 4 / 5(C61-C119), NT - 4 / 5(C61-C78), NT - 4 / 5(C78-C119), NT - 4 / 5(C61-C90), NT - 4 / 5(R60-C78), NT - 4 / 5(K62-C119), NT - 4 / 5(K62-K91), NT - 4 / 5(R79-R98), NT - 4 / 5(R83-R93), NT - 4 / 5(T101-R111), NT - 4 / 5(G1-C121) VLTVKRVRR (SEQ ID NO:4), NT - 4 / 5(V40-C121) VLTVKRVRR (SEQ ID NO:5), NT - 4 / 5(V40-C121) SLTIKRIRA (SEQ ID NO:6), NT - 4 / 5(V40-C121) TLSRKAGRRA (SEQ ID NO:7), DDDSPIARRGEISVCDSVSDWVSAPDKDTAVDIKGDDVMVLKKVGINHSV (SEQ ID NO:8), and NT - 4 / 5(V40-C121)...

...neuronal damage, loss or degeneration, comprising administering to the neuron an effective amount of a **trkB** or **trkC** agonist, wherein said agonist is (a) a NT - 4 / 5 (SEQ ID NO:1) variant comprising one to five amino acid alterations selected from...

...single amino acid or deletion of the indicated span of residues, inclusive; or (b) a NT - 4 / 5 (SEQ ID NO:1) fragment selected from the group consisting of NT - 4 / 5(R83-Q94), NT - 4 / 5(G1-C61), NT - 4 / 5(G1-C17), NT - 4 / 5(C17-C61), NT - 4 / 5(C17-C78), NT - 4 / 5(C17-C90), NT - 4 / 5(C17-C119), NT - 4 / 5(C17-C121), NT - 4 / 5(R11-R27), NT - 4 / 5(R11-R34), NT - 4 / 5(R34-R53), NT - 4 / 5(C61-C78), NT - 4 / 5(R53-C61), NT - 4 / 5(C61-C119), NT - 4 / 5(C61-C78), NT - 4 / 5(C78-C119), NT - 4 / 5(C61-C90), NT - 4 / 5(R60-C78), NT - 4 / 5(K62-C119), NT - 4 / 5(K62-K91), NT - 4 / 5(R79-R98), NT - 4 / 5(R83-R93), NT - 4 / 5(T101-R111), NT - 4 / 5(G1-C121) VLTVKRVRR (SEQ ID NO:4), NT - 4 / 5(V40-C121) VLTVKRVRR (SEQ ID NO:5), NT - 4 / 5(V40-C121) SLTIKRIRA (SEQ ID NO:6), NT - 4 / 5(V40-C121) TLSRKAGRRA (SEQ ID NO:7), DDDSPIARRGEISVCDSVSDWVSAPDKDTAVDIKGDDVMVLKKVGINHSV (SEQ ID NO:8), and NT - 4 / 5(V40-C121)...

13/3,K,AB/16 (Item 3 from file: 340)
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IFI Publication Control No: 0039692
Document Type: C

**HUMAN TRK RECEPTORS AND NEUROTROPHIC FACTOR INHIBITORS; BONDING A
NEUROTROPHIN WITH A CHIMERIC POLYPEPTIDE COMPRISING AN IMMUNOGLOBULIN
CONSTANT DOMAIN SEQUENCE FUSED TO A POLYPEPTIDE SEQUENCE FROM A SECOND
IMMUNOGLOBULIN-LIKE DOMAIN SEQUENCE OF HUMAN TYROSINE KINASES RECEPTOR**
Inventors: Presta Leonard G (US); Shelton David L (US); Ufer Roman (US)
Assignee: Genentech Inc
Assignee Code: 07579

Attorney, Agent or Firm: Knobbe, Martens, Olson & Bear, LLP

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Priority Applic(No,Date): US 98156923 19980918; US 94359705

19941220; US 94215139 19940318; US 94286846 19940805

Abstract: The invention concerns human **trkB** and trkC receptors and their functional derivatives. The invention further concerns immunoadhesins comprising trk receptor sequences fused to immunoglobulin sequences.

Publication (No,Kind,Date), Applic (No,Date):
... 20001128

Abstract: The invention concerns human **trkB** and trkC receptors and their functional derivatives. The invention further concerns immunoadhesins comprising trk receptor...

Exemplary Claim:

...to a polypeptide sequence comprising a second immunoglobulin-like domain sequence of human trkA, human **trkb**, or human trk C, wherein said chimeric polypeptide binds said neurotrophin.

Non-exemplary Claims:

...The method of claim 2 wherein inhibition of said biological activity is the inhibition of **tumor** development...

...5. The method of claim 1 wherein said neurotrophin is NT-3, **NT - 4** or **NT - 4 /5**, and said second immunoglobulin-like domain sequence is that in human **trkB**.

...

...claim 5 wherein inhibition of said biological activity is the inhibition aberrant neuron sprouting or **tumor** development...

...7 wherein inhibition of said biological activity is the inhibition of aberrant neuron sprouting or **tumor** development.

13/3,K,AB/17 (Item 4 from file: 340)

DIALOG(R) File 340:CLAIMS(R)/US Patent
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IFI Publication Control No: 0030789
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TREATMENT OF BALANCE IMPAIRMENTS; ADMINISTERING TRKB OR TRKC AGONIST

Inventors: Gao Wei-Qiang (US)
Assignee: Genentech Inc
Assignee Code: 07579
Attorney, Agent or Firm: Knobbe, Martens, Olson & Bear LLP
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Document Type: CERTIFICATE OF CORRECTION

Certificate of Correction Date: 20021001
Priority Applic(No,Date): US 95581662 19951229

Abstract: Compositions and methods are provided for prophylactic or therapeutic treatment of balance impairments involving neuronal damage, loss, or degeneration, preferably of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 /5**.

...ADMINISTERING TRKB OR TRKC AGONIST

Publication (No,Kind,Date), Applic (No,Date):
... 20000919

Abstract: ...of vestibular ganglion neurons, in an animal by administration of an effective amount of a **trkB** or **trkC** agonist, particularly a neurotrophin, more preferably **NT - 4 /5**.

Exemplary Claim:

...administering to a mammal in need of such treatment a therapeutically effective amount of a **trkB** or **trkC** agonist.

Non-exemplary Claims:

...The method of claim 2, wherein the agonist is selected from the group consisting of **NT - 4 /5**, BDNF and NT-3...

...4. The method of claim 3, wherein the agonist is **NT - 4 /5**...

...11. The method of claim 6, wherein the **trkB** or **trkC** agonist is administered prior to administration of an ototoxin...

...The method of claim 1, which further comprises administering an effective amount of a second **trkB** or **trkC** agonist...

...15. A method of assaying for a **trkB** or **trkC** agonist that provides vestibular ganglion neuron protection or survival from an ototoxin, comprising, culturing a utricle explant, administering a **trkB** or **trkC** agonist to the culture, administering an ototoxin to the culture, and determining the amount of protection or survival compared to a control culture to which the **trkB** or **trkC** agonist was not administered...

...A pharmaceutical composition, comprising a pharmaceutical agent capable of ototoxin-induced balance impairment and a **trkB** or **trkC** agonist in an amount ...20. The composition of claim 19, wherein the agonist is

NT - 4 /5...

...administration of an aminoglycoside antibiotic, the improvement comprising administering a therapeutically effective amount of a **trkB** or **trkC** agonist to the mammal in need of such treatment to reduce or prevent...

...The method of claim 23, wherein the agonist is selected from the group consisting of NT - 4 /5, BDNF and NT-3...

...25. The method of claim 24, wherein the agonist is NT - 4 /531. An improved method for treating a mammal undergoing treatment of **cancer** by administration of a chemotherapeutic compound, the improvement comprising administering a therapeutically effective amount of a **trkB** or **trkC** agonist to the mammal in need of such treatment to reduce or prevent...

...The method of claim 32, wherein the agonist is selected from the group consisting of NT - 4 /5, BDNF or NT-3...

...34. The method of claim 33, wherein the agonist is NT - 4 /5...

...causing neuronal damage, loss, or degeneration, comprising administering to the neuron an effective amount of **trkB** or **trkC** agonist...

...The method of claim 39, wherein the agonist is selected from the group consisting of NT - 4 /5, BDNF or NT-3...

...41. The method of claim 40, wherein the agonist is NT - 4 /5...48. The method of claim 38, wherein the **trkB** or **trkC** agonist is administered prior to exposure to the ototoxin...

...The composition of claim 48, wherein the agonist is selected from the group consisting of NT - 4 /5, BDNF and NT-3.

? log off

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10oct06 10:58:16 User231882 Session D1724.2
$11.93      3.510 DialUnits File155
    $2.42   11 Type(s) in Format  4 (UDF)
    $2.42   11 Types
$14.35 Estimated cost File155
    $11.43    1.904 DialUnits File55
        $2.20   1 Type(s) in Format  3 (UDF)
        $2.20   1 Type(s) in Format  4 (UDF)
        $4.40    2 Types
$15.83 Estimated cost File55
    $56.02    2.387 DialUnits File34
$56.02 Estimated cost File34
    $27.99    1.192 DialUnits File434
$27.99 Estimated cost File434
    $52.76    3.015 DialUnits File340
        $12.52   4 Type(s) in Format  5 (UDF)
        $12.52   4 Types
$65.28 Estimated cost File340
    OneSearch, 5 files, 12.008 DialUnits FileOS
    $2.93 TELNET
$182.40 Estimated cost this search
$182.48 Estimated total session cost 12.223 DialUnits
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Logoff: level 05.12.03 D 10:58:16

You are now logged off

Inverse expression of neurotrophins and neurotrophin receptors at the invasion front of human melanoma brain metastases

AUTHOR: Marchetti Dario (Reprint); McCutcheon Ian E; Ross Merrick J; Nicolson Garth L

AUTHOR ADDRESS: Dep. Tumor Biol., Box 108, M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA**USA

JOURNAL: International Journal of Oncology 7 (1): p87-94 1995 1995

ISSN: 1019-6439

DOCUMENT TYPE: Article

RECORD TYPE: Abstract

LANGUAGE: English

ABSTRACT: Neurotrophins (NT), such as nerve growth factor (NGF), stimulate the growth and differentiation of several neuronal subpopulations in a distinct yet overlapping manner. Brain-metastatic human melanoma cells overexpress p75-NTR, the low-affinity neurotrophin receptor, and treatment of brain-metastatic cells with NGF stimulates extracellular matrix invasion and production of degradative enzymes in relation to the cellular expression of p75-NTR. Although human melanoma cells express high affinity neurotrophin receptors, such as TrkC (the putative receptor for NT-3), they do not express TrkA, the high-affinity NGF receptor. Using digoxigenin-labeled sense/antisense riboprobes against human p75-NTR and NGF for in situ hybridization, we determined whether the expression of p75-NTR and NGF mRNAs are related to brain metastasis of human melanoma. We **detected** p75-NTR mRNA at the invasion front of human melanoma brain metastases, whereas p75-NTR expression was not found in adjacent tissues. In contrast, human NGF mRNA levels were increased in tissues surrounding the melanoma lesions, supporting the notion that NGF and NT are important in determining melanoma brain-metastatic microenvironment. Using antibodies specific to p75-NTR, TrkC, NGF and related NT we found high but heterogeneous levels of p75-NTR and TrkC expression in **malignant** melanomas metastatic to the brain. Lower levels of expression were found in primary melanomas or in metastatic melanomas to sites other than brain. Additionally, we found elevated levels of synthesis of NGF and NT-3 but not brain-derived neurotrophic factor (BDNF) or **NT - 4 / 5** in the brain tissues surrounding melanoma lesions. These studies support a role for NT and their receptors in the progression of melanomas to the brain-metastatic phenotype.

1995

...ABSTRACT: of p75-NTR and NGF mRNAs are related to brain metastasis of human melanoma. We **detected** p75-NTR mRNA at the invasion front of human melanoma brain metastases, whereas p75-NTR...

...related NT we found high but heterogeneous levels of p75-NTR and TrkC expression in **malignant** melanomas metastatic to the brain. Lower levels of expression were found in primary melanomas or...

...of synthesis of NGF and NT-3 but not brain-derived neurotrophic factor (BDNF) or **NT - 4 / 5** in the brain tissues surrounding melanoma lesions. These studies support a role for NT and...

10/3,K,AB/31 (Item 1 from file: 34)

DIALOG(R) File 34:SciSearch(R) Cited Ref Sci

(c) 2006 The Thomson Corp. All rts. reserv.

10862038 Genuine Article#: 575HR Number of References: 17

Title: MR imaging of pancreatic lesions with Mn-DPDP. A histopathologic correlation (ABSTRACT AVAILABLE)

Author(s): Dobritz M (REPRINT) ; Fellner FA; Baum U; Nomayr A; Lell M; Klein P; Papadopoulos T; Bautz W

Corporate Source: Tech Univ Munich, Klinikum Rechts Isar, Inst Rontgendiagnost, Ismaninger Str 22/D-81675 Munich//Germany/ (REPRINT); Univ Erlangen Nurnberg, Inst Diag Radiol, D-8520 Erlangen//Germany//; Univ Erlangen Nurnberg, Chirurg Klin & Poliklin, D-8520 Erlangen//Germany//; Univ Erlangen Nurnberg, Inst Pathol Anat, D-8520 Erlangen//Germany/

Journal: ROFO-FORTSCHRITTE AUF DEM GEBIET DER RONTGENSTRAHLEN UND DER BILDGEBENDEN VERFAHREN, 2002, V174, N7 (JUL), P893-897

ISSN: 1438-9029 Publication date: 20020700

Publisher: GEORG THIEME VERLAG KG, RUDIGERSTR 14, D-70469 STUTTGART, GERMANY

Language: German Document Type: ARTICLE

Abstract: Purpose: To examine the **diagnostic** accuracy of pancreatic lesions using mangafodipir-trisodium (Mn-DPDP) enhanced MR imaging. The imaging results were correlated with the histopathological results. Material and Methods: 40 patients with suspicion of pancreatic carcinoma were examined with MRI before and after i.v. administration of Mn-DPDP (Philips Gyroscan ACS **NT** 1.5 T, phased array body-coil: TSE T-2 with and without SPIR, TR 2000 ms, TE 120ms; FFE T-1 breathhold, TR 115 ms, TE 4.6 ms; MRCP, TR 6000 ms, TE 1200 ms; Teslascan i.v. 5 mmol Mn/kg; FFE T-1 breathhold SPIR, TR 140 ms, TE 4.6 ms). Two observers evaluated in consensus the number and characteristics of focal pancreatic lesions. The MR findings were correlated with histopathological findings retrospectively. Results: The following lesions were found: adenocarcinoma (19), pancreatitis (8), adenocarcinoma within pancreatitis (3), insulinoma (2), hematoma (1), papillitis stenosis (1), signet ring cell carcinoma (1), metastasis of rectal carcinoma (1), papillary mesothelioma (1). In three patients there was no pathological finding. Mn-DPDP enhanced MRI showed a sensitivity of 100% and a specificity of 56%. Conclusion: Mn-DPDP enhanced MRI in conjunction with MRCP showed a high sensitivity for the **detection** of pancreatic lesions. However, the specificity is low, thus recommending Mn-DPDP enhanced MRI only as a complementary imaging method.

, 2002

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...examined with MRI before and after i.v. administration of Mn-DPDP (Philips Gyroscan ACS **NT** 1.5 T, phased array body-coil: TSE T-2 with and without SPIR, TR 2000 ms...

...Conclusion: Mn-DPDP enhanced MRI in conjunction with MRCP showed a high sensitivity for the **detection** of pancreatic lesions. However, the specificity is low, thus recommending Mn-DPDP enhanced MRI only...

...Identifiers--GADOLINIUM CHELATE; MANGANESE-DPDP; HELICAL CT; **TUMORS**; ENHANCEMENT; **CANCER**

10/3,K,AB/32 (Item 2 from file: 34)
DIALOG(R) File 34:SciSearch(R) Cited Ref Sci
(c) 2006 The Thomson Corp. All rts. reserv.

06168410 Genuine Article#: XZ350 Number of References: 71

Title: Optimization of the isolation and amplification of RNA from formalin-fixed, paraffin-embedded tissue: The armed forces institute of pathology experience and literature review (ABSTRACT AVAILABLE)

Author(s): Krafft AE (REPRINT) ; Duncan BW; Bijwaard KE; Taubenberger JK; Lichy JH

Corporate Source: ARMED FORCES INST PATHOL, DEPT CELLULAR PATHOL, DIV MOL PATHOL/WASHINGTON//DC/20306 (REPRINT)

Journal: MOLECULAR DIAGNOSIS, 1997 , V2, N3 (SEP), P217-230

ISSN: 1084-8592 Publication date: 19970900

Publisher: CHURCHILL LIVINGSTONE INC MEDICAL PUBLISHERS, 650 AVENUE OF THE AMERICAS, NEW YORK, NY 10011

Language: English Document Type: REVIEW

Abstract: Background: RNA is extensively degraded by routine formalin fixation to fragments averaging 200 nucleotides (nt). Several methods for the recovery of amplifiable RNA from formalin-fixed, paraffin-embedded tissue have been described; however, a universally accepted approach in a clinical molecular **diagnostic** laboratory has not yet emerged.

Methods and Results: Amplifiable RNA can be recovered with high efficiency from all types of formalin-fixed, paraffin-embedded tissue using proteinase K digestion, either a phenol-chloroform or an acidic guanidinium thiocyanate-phenol chloroform extraction step, and isopropanol precipitation in the presence of glycogen. Designing primers to **detect** a small target was critical for consistent RNA amplification in the following assays, with the target sizes indicated: hepatitis C virus, 169 nt; morbillivirus, 78 nt; influenza virus, 113 nt; the npm-alk fusion product resulting from t(2; 5) translocation, 175 nt ; and the bcr-abl fusion product resulting from t(9;22) translocation, 93 or 168 nt.

Conclusions: With use of beta-2-mi

Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina.

Ghazi-Nouri S M S; Assi A; Limb G A; Scott R A H; von Bussmann K; Humphrey I; Luthert P J; Charteris D G

Moorfields Eye Hospital, London, UK. snouri@doctors.org.uk

British journal of ophthalmology (England) Apr 2003 , 87 (4) p488-92, ISSN 0007-1161--Print Journal Code: 0421041

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

AIMS: To investigate the staining pattern of neurotrophin-3 (NT3), neurotrophin-4 (**NT4**), and brain derived neurotrophic factor (BDNF) as well as glial fibrillary acid protein (GFAP) and CD68 in lasered human retina. METHODS: Retinal laser photocoagulation was performed on four patients (two males, two females) with choroidal **malignant** melanoma 1-6 days before enucleation. Three other enucleated eyes with **malignant** melanoma and three normal cadaveric donor eyes were used as controls. Immunohistochemistry was performed to investigate the pattern of staining of NT3, **NT4** , BDNF, GFAP, and CD68 in 7 mm sections of fixed specimens. RESULTS: Expression of **NT4** was **detected** in the inner and outer nuclear layers of all the retinal sections examined but no NT3 and BDNF staining was seen. **NT4** staining was found to be less intense in lasered and melanoma controls compared to normal cadaveric donor retinas. There was an upregulation of GFAP expression in both lasered and control eyes with melanoma in comparison with normal controls. CD68 staining was only observed in retinal pigment epithelium and choroid of lasered eyes. CONCLUSION: **NT4** is expressed in inner and outer nuclear layers of normal human retina and its expression is downregulated following laser photocoagulation. This occurs in parallel with an increased expression of GFAP suggesting that reactive changes in Muller cells may be responsible for reduced **NT4** staining. Expression of CD68 at the site of laser injury

is consistent with a wound healing process as a response to local damage.

... 2003 ,

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... METHODS: Retinal laser photocoagulation was performed on four patients (two males, two females) with choroidal **malignant** melanoma 1-6 days before enucleation. Three other enucleated eyes with **malignant** melanoma and three normal cadaveric donor eyes were used as controls. Immunohistochemistry was performed to investigate the pattern of staining of NT3, **NT4** , BDNF, GFAP, and CD68 in 7 mm sections of fixed specimens. RESULTS: Expression of **NT4** was **detected** in the inner and outer nuclear layers of all the retinal sections examined but no NT3 and BDNF staining was seen. **NT4** staining was found to be less intense in lasered and melanoma controls compared to normal...

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... expression of GFAP suggesting that reactive changes in Muller cells may be responsible for reduced **NT4** staining. Expression of CD68 at the site

of laser injury is consistent with a wound...

10/3,K,AB/3 (Item 3 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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14215104 PMID: 12637152

Epstein-Barr virus and human hepatocellular carcinoma.

Akhter Shamim; Liu Huifeng; Prabhu Ramesh; DeLucca Cynthia; Bastian Frank
; Garry Robert F; Schwartz Myron; Thung Swan N; Dash Srikanta

Department of Pathology and Laboratory Medicine, Tulane University Health
Sciences Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA.

Cancer letters (Ireland) Mar 20 2003 , 192 (1) p49-57, ISSN
0304-3835--Print Journal Code: 7600053

Contract/Grant No.: CA54576; CA; NCI; CA89121; CA; NCI

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Recent studies suggest that Epstein-Barr virus (EBV) may act as a helper
virus for the development of hepatocellular carcinoma by promoting
replication of the hepatitis C virus (HCV) in the infected liver.

Detection of EBV DNA in a high percentage of HCV-positive human
hepatocellular carcinomas (HCC) from Japanese patients has supported this
concept. In order to determine whether EBV infection is associated with
HCC, we examined paraffin-embedded tissues from 31 cases of non-cirrhotic
livers with hepatocellular carcinoma for the presence of EBV, HCV and
hepatitis B virus (HBV) infection. RNA prepared from **tumor** samples were
used as a template for reverse transcription followed by double-nested PCR
with primers for the 5' untranslated region (**NT**) of HCV. DNA extracts
of **tumor** samples were tested by single polymerase chain reaction for the
detection of EBV and HBV (X- and/or S-gene) DNA sequences. To control for
nucleic acid integrity, all **tumor** samples were amplified for human
beta-globin DNA by polymerase chain reaction and subjected to Southern blot
hybridization. None of the cases was found to be positive for EBV. Ten HCC
cases (32%) tested positive for HCV and 12 HCC cases (38%) tested positive
for HBV. Six of the surveyed patients had nucleic acids of both HCV and HBV
in their **tumor** tissue. All HCC **tumor** samples were positive for
beta-globin. Our study shows that HCV and HBV infections, but not EBV
infection, are associated with hepatocarcinogenesis in non-cirrhotic
livers. Other unknown risk factors seem to be in effect in the development
of hepatocellular carcinoma in non-cirrhotic livers.


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s nt4 or (nt(w)4?) or (nt(2n)5)
>>>File 155 processing for 4? stopped at 4META
>>>File 55 processing for 4? stopped at 4G20010
>>>File 34 processing for 4? stopped at 4D8A4A4
>>>File 434 processing for 4? stopped at 42500
>>>File 340 processing for 4? stopped at 4BROMOBENZYLOXYCAROBNYL
Processing

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      49451 NT
      9928712 4?
      2004 NT(W)4?
      49451 NT
      9942455 5
      4231 NT(2N)5
s1 5736 NT4 OR (NT(W)4?) OR (NT(2N)5)
? s cancer? or tumor? or malignan?
      1742492 CANCER?
      2094993 TUMOR?
      630722 MALIGNAN?
s2 3432215 CANCER? OR TUMOR? OR MALIGNAN?
? s s1 and s2
      5736 S1
      3432215 S2
s3 451 S1 AND S2
? s detect? or diagnos?
Processing
      3486928 DETECT?
      3848381 DIAGNOS?
s4 6807923 DETECT? OR DIAGNOS?
? s s3 and s4
      451 S3
      6807923 S4
s5 118 S3 AND S4
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>>>Duplicate detection is not supported for File 340.

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>>>Records from unsupported files will be retained in the RD set.

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Processing

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s7 58 S6 AND PY<=2003
? s cell(w)line??
Processing

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s8 1045564 CELL(W)LINE??
? s s7 and s8

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      58 S7
      1045564 S8
s9 16 S7 AND S8
? s s7 not s8

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      58 S7
      1045564 S8
s10 42 S7 NOT S8
? t s10/3,k,ab/1-42

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10/3,K,AB/1 (Item 1 from file: 155)
DIALOG(R) File 155:MEDLINE(R)
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14706476 PMID: 15600203

[Preliminary study on correlation between double-contrast and serum 5'-nucleotidase of gastric carcinoma]

Yu Jianqun; Liao Fangyi; Chen Manling; Yang Kaiyu
Department of Radiology, West China Hospital, Sichuan China University, Chengdu 610041, China.

Sichuan da xue xue bao. Yi xue ban = Journal of Sichuan University. Medical science edition (China) Jan 2003 , 34 (1) p128-30, ISSN 1672-173X--Print Journal Code: 101162609

Publishing Model Print

Document type: Journal Article ; English Abstract

Languages: CHINESE

Main Citation Owner: NLM

Record type: MEDLINE; Completed

OBJECTIVE: To determine the relationship between double-contrast manifestation and 5'-nucleotidase (5'-NT) of gastric carcinoma. **METHODS:** Serum specimens were taken from 41 cases with gastric carcinoma documented by double-contrast visualization and pathology. 5'-NT activity was determined by spectrophotometry. 40 healthy subjects were recruited and studied as normal controls. **RESULTS:** The serum level of 5'-NT (1.14 u/ml) in gastric carcinoma group was lower than that of control group (2.60 u/ml) ($P < 0.05$). Anatomically, it was found that the 5'-NT level of gastric carcinoma gradually increased as the sites of the tumor lined up from the orifice (0.98 u/ml) to the antrum (1.28 u/ml) via the body (1.20 u/ml). 5'-NT of the ulcerative type (0.96 u/ml) was lower than that of the fungating type (1.12 u/ml) or the infiltrating type (1.40 u/ml). 5'-NT (1.53 u/ml) of stage IV gastric carcinoma was higher than that of stage I (0.83 u/ml), stage II (0.98 u/ml) and stage III (1.03 u/ml) ($P < 0.05$). **CONCLUSION:** The change of 5'-NT from gastric carcinoma was correlated with the site, type or stage shown on the double-contrast images of the tumor .

... 2003 ,

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...ml) and stage III (1.03 u/ml) ($P < 0.05$). **CONCLUSION:** The change of 5'-NT from gastric carcinoma was correlated with the site, type or stage shown on the double-contrast images of the tumor .

; Adenocarcinoma--ldiagnosis --DI; Adenocarcinoma--enzymology--EN; Adenocarcinoma--radiography--RA; Adult; Aged; English Abstract; Humans;

Middle Aged; Stomach Neoplasms-- diagnosis --DI; Tumor Markers,
Biological--blood--BL
Chemical Name: Contrast Media; Tumor Markers, Biological;
5'-Nucleotidase

10/3,K,AB/2 (Item 2 from file: 155)
DIALOG(R) File 155:MEDLINE(R)
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14218987 PMID: 12642316

Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina.

Ghazi-Nouri S M S; Assi A; Limb G A; Scott R A H; von Bussmann K;
Humphrey I; Luthert P J; Charteris D G

Moorfields Eye Hospital, London, UK. snouri@doctors.org.dk

British journal of ophthalmology (England) Apr 2003 , 87 (4)
p488-92, ISSN 0007-1161--Print Journal Code: 0421041

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

AIMS: To investigate the staining pattern of neurotrophin-3 (NT3), neurotrophin-4 (**NT4**), and brain derived neurotrophic factor (BDNF) as well as glial fibrillary acid protein (GFAP) and CD68 in lasered human retina. METHODS: Retinal laser photocoagulation was performed on four patients (two males, two females) with choroidal **malignant** melanoma 1-6 days before enucleation. Three other enucleated eyes with **malignant** melanoma and three normal cadaveric donor eyes were used as controls. Immunohistochemistry was performed to investigate the pattern of staining of NT3, **NT4** , BDNF, GFAP, and CD68 in 7 mm sections of fixed specimens. RESULTS: Expression of **NT4** was **detected** in the inner and outer nuclear layers of all the retinal sections examined but no NT3 and BDNF staining was seen. **NT4** staining was found to be less intense in lasered and melanoma controls compared to normal cadaveric donor retinas. There was an upregulation of GFAP expression in both lasered and control eyes with melanoma in comparison with normal controls. CD68 staining was only observed in retinal pigment epithelium and choroid of lasered eyes. CONCLUSION: **NT4** is expressed in inner and outer nuclear layers of normal human retina and its expression is downregulated following laser photocoagulation. This occurs in parallel with an increased expression of GFAP suggesting that reactive changes in Muller cells may be responsible for reduced **NT4** staining. Expression of CD68 at the site of laser injury is consistent with a wound healing process as a response to local damage.

... 2003 ,

AIMS: To investigate the staining pattern of neurotrophin-3 (NT3), neurotrophin-4 (**NT4**), and brain derived neurotrophic factor (BDNF) as well as glial fibrillary ac

Relevant genomics of neurotensin receptor in cancer (ABSTRACT AVAILABLE)

Author(s): Elek J; Pinzon W; Park KH; Narayanan R (REPRINT)

Corporate Source: FLORIDA ATLANTIC UNIV,CTR MOL BIOL & BIOTECHNOL, 777 GLADES RD/BOCA RATON//FL/33431 (REPRINT); FLORIDA ATLANTIC UNIV,CTR MOL BIOL & BIOTECHNOL/BOCA RATON//FL/33431; FLORIDA ATLANTIC UNIV,DEPT BIOL/BOCA RATON//FL/33431

Journal: ANTICANCER RESEARCH, 2000 , V20, N1A (JAN-FEB), P53-58

ISSN: 0250-7005 Publication date: 20000100

Publisher: INT INST ANTICANCER RESEARCH, EDITORIAL OFFICE 1ST KM KAPANDNTIOU-KALAMOU RD KAPANDRITI, POB 22, ATHENS 19014, GREECE

Language: English Document Type: ARTICLE

Abstract: The expressed sequence tag (EST) databases are an attractive starting point for gene discovery for diseases like **cancer** . Validation of gene targets from these sequences (both known and novel) in **cancers** requires a comprehensive expression profiling. We identified from the **Cancer** Gene Anatomy Project database (CGAP), a hit called neurotensin receptor (NT-r) that was expressed in the pancreatic **cancer** cDNA libraries. Neurotensin (NT), a neuroendocrine peptide, exerts trophic effects in vivo and stimulates the growth of **cancer** -derived cell lines in vitro. High affinity neurotensin receptors (NT-r) are expressed in **cancer** -derived cell lines and in some primary **tumors** . To elate, a comprehensive expression profile of the NT-r in diverse **cancers** and normal **tissues** has not been reported. A **cancer** -selective expression of **NT - 4** ; if demonstrable, may provide a basis for a diagnostic and potential therapeutic utility. We demonstrate that the NT-r is expressed in a variety of **cance**

Neurotrophins and Trk receptors in human pancreatic ductal adenocarcinoma: expression patterns and effects on in vitro invasive behavior.

Miknyoczki S J; Lang D; Huang L; Klein-Szanto A J; Dionne C A; Ruggeri B
A

Department of Pathology, MCP-Hahnemann University, Philadelphia, PA, USA.
International journal of cancer. Journal international du cancer (UNITED STATES) May 5 1999 , 81 (3) p417-27, ISSN 0020-7136--Print
Journal Code: 0042124

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

The aggressive and highly metastatic behavior observed in pancreatic ductal adenocarcinoma (PDAC) may be due to autocrine and/or paracrine interactions (**tumor** /stromal) involving altered expression of peptide growth factors and their corresponding receptors. The neurotrophin (NT) growth factor family and their cognate receptors have been demonstrated to play a role in the invasiveness, chemotactic behavior and **tumor** cell survival of both neuronal and non-neuronal **cancers** . We hypothesized that aberrant expression of the NTs and/or the Trk receptors may contribute to the **malignant** phenotype of PDAC, specifically **tumor** cell invasiveness, through autocrine and/or paracrine interactions. In this study, we examined the expression of NTs, Trks and p75NGFR by immunohistochemical and in situ hybridization analyses in both normal (n=14) and neoplastic pancreas (n=47) and PDAC-derived cell lines (n=6). Further, we evaluated the effects of various NTs on the in vitro invasive and chemotactic behavior on 6 human PDAC-derived cell lines in a modified Boyden chamber ass

Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina.

Ghazi-Nouri S M S; Assi A; Limb G A; Scott R A H; von Bussmann K; Humphrey I; Luthert P J; Charteris D G

Moorfields Eye Hospital, London, UK. snouri@doctors.org.uk

British journal of ophthalmology (England) Apr 2003 , 87 (4)
p488-92, ISSN 0007-1161--Print Journal Code: 0421041

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

AIMS: To investigate the staining pattern of neurotrophin-3 (NT3), neurotrophin-4 (**NT4**), and brain derived neurotrophic factor (BDNF) as well as glial fibrillary acid protein (GFAP) and CD68 in lasered human retina. METHODS: Retinal laser photocoagulation was performed on four **patients** (two males, two females) with choroidal **malignant** melanoma 1-6 days before enucleation. Three other enucleated eyes with **malignant** melanoma and three normal cadaveric donor eyes were used as controls. Immunohistochemistry was performed to investigate the pattern of staining of NT3, **NT4** , BDNF, GFAP, and CD68 in 7 mm sections of fixed specimens. RESULTS: Expression of **NT4** was detected in the inner and outer nuclear layers of all the retinal sections examined but no NT3 and BDNF staining was seen. **NT4** staining was found to be less intense in lasered and melanoma controls compared to normal cadaveric donor retinas. There was an upregulation of GFAP expression in both lasered and cont

Expression of NGF family and their receptors in gastric carcinoma: a cDNA microarray study.

Du Jian-Jun; Dou Ke-Feng; Peng Shu-You; Qian Bing-Zhi; Xiao Hua-Sheng; Liu Feng; Wang Wei-Zhong; Guan Wen-Xian; Gao Zhi-Qing; Liu Ying-Bin; Han Ze-Guang

Department of General Surgery, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shanxi Province, China.

World journal of gastroenterology - WJG (China) Jul 2003 , 9 (7) p1431-4, ISSN 1007-9327--Print Journal Code: 100883448

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

AIM: To investigate the expression of NGF family and their receptors in gastric carcinoma and normal gastric mucosa, and to elucidate their effects on gastric carcinoma. METHODS: RNA of gastric **cancer tissues** and normal gastric **tissues** was respectively isolated and mRNA was purified. Probes of both mRNA reverse transcription product cDNAs labeled with alpha-(33)P dATP were respectively hybridized with Atlas Array membrane where NGF and their family genes were spotted on. Hybridized signal images were scanned on phosphor screen with ImageQuant 5.1 software after hybridization. Normalized values on spots were analyzed with ArrayVersion 5.0 software. Differential expression of NGF family and their receptors mRNA was confirmed between hybridized Atlas Array membranes of gastric **cancer tissues** and normal gastric mucosa, then their effects on gastric carcinoma were investigated. RESULTS: Hybridization signal images on Atlas Array membrane appeared in a lower level of nonspecific hybridization. Both of NGF family and their receptors Trk family mRNA were expressed in gastric **cancer** and normal gastric mucosa. But adversely up-regulated expression in other **tissues** and organs. NGF, BDGF, NT-3, **NT - 4 /5**, NT-6 and TrkA, B and C were down-regulated simultaneously in gastric carcinoma in comparison with normal gastric mucosa. Degrees of down-regulation in NGF family were greater than those in their receptors Trk family. Down-regulation of NT-3 and BDGF was the most significant, and TrkC down-regulation level was the lowest in receptors Trk family. CONCLUSION: Down-regulated expression of NGF family and their receptors Trk family mRNA in gastric **cancer** is confirmed. NGF family and their receptors Trk family probably play a unique role in gastric **cancer** cell apoptosis by a novel Ras or Raf signal transduction pathway. Their synchronous effects are closely associated with occurrence and development of gastric carcinoma induced by reduction of signal transduction of programmed cell death.

... 2003 ,

... normal gastric mucosa, and to eluc

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s nt4 or (nt(w)4?)
>>>File 155 processing for 4? stopped at 4META
>>>File 55 processing for 4? stopped at 4G20010
>>>File 34 processing for 4? stopped at 4D8A4A4
>>>File 434 processing for 4? stopped at 42500
>>>File 340 processing for 4? stopped at 4BROMOBENZYLOXYCAROBNYL
      538 NT4
      49451 NT
      9928712 4?
      2004 NT(W)4?
S1 2474 NT4 OR (NT(W)4?)
? s cancer? or malignan?or tumor?
      1742492 CANCER?
      0 MALIGNAN?OR TUMOR?
S2 1742492 CANCER? OR MALIGNAN?OR TUMOR?
? s s1 and s2
      2474 S1
      1742492 S2
S3 51 S1 AND S2
? s cancer? or tumor? or malignan?
      1742492 CANCER?
      2094993 TUMOR?
      630722 MALIGNAN?
S4 3432215 CANCER? OR TUMOR? OR MALIGNAN?
? s s1 and s4
      2474 S1
      3432215 S4
S5 147 S1 AND S4
? rd

>>>Duplicate detection is not supported for File 340.

>>>Records from unsupported files will be retained in the RD set.
S6 99 RD (unique items)
? s necrosis
S7 479099 NECROSIS
? s s6 not s7
      99 S6
      479099 S7
S8 72 S6 NOT S7
? rd

>>>Duplicate detection is not supported for File 340.

>>>Records from unsupported files will be retained in the RD set.
S9 72 RD (unique items)
? s s9 and py<=2003
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      47242220 PY<=2003
S10 62 S9 AND PY<=2003
? s trkB
S11 6358 TRKB
? s tissue?? or patient??
Processing
Processing
      2428597 TISSUE??
      6810840 PATIENT??
S12 8709968 TISSUE?? OR PATIENT??
? s s10 and s12

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62 S10
8709968 S12
S13 31 S10 AND S12
? t s13/3,k,ab/1-31

13/3,K,AB/1 (Item 1 from file: 155)
DIALOG(R) File 155:MEDLINE(R)
(c) format only 2006 Dialog. All rts. reserv.

14582503 PMID: 14613990

Reverse transcription-PCR analysis of laser-captured cells points to potential paracrine and autocrine actions of neurotrophins in pancreatic cancer .

Ketterer Knut; Rao Shyam; Friess Helmut; Weiss John; Buchler Markus W; Korc Murray

Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of California, Irvine, California 92697, USA.

Clinical cancer research - an official journal of the American Association for Cancer Research (United States) Nov 1 2003 , 9 (14) p5127-36, ISSN 1078-0432--Print Journal Code: 9502500

Contract/Grant No.: CA-40162; CA; NCI

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

PURPOSE: Neurotrophins (NTs) can stimulate cell proliferation and differentiation in various cell types, and play a role in certain human cancers . In this study we analyzed the ex

ssociation of neurotrophin receptor expression and differentiation in human neuroblastoma.

Hoehner J C; Olsen L; Sandstedt B; Kaplan D R; Pahlman S

Department of Pathology, Uppsala University Hospital, Sweden.

American journal of pathology (UNITED STATES) Jul 1995 , 147 (1)
p102-13, ISSN 0002-9440--Print Journal Code: 0370502

Contract/Grant No.: N01-CO046000; CO; NCI

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Interactions of the trk family of tyrosine kinase receptors with neurotrophins result in growth and maturational changes in neuronal cells. The continued progression, maturation, or regression of neuroblastoma, an embryonal, sympathetic nervous system-derived tumor of infants and children, might be governed by neurotrophic influences. Immunocytochemistry was utilized to evaluate TrkA, TrkB, and TrkC protein expression at the cellular level in the developing human fetal sympathetic nervous system and in a selection of neuroblastoma tumor specimens. TrkA and TrkC expression was identified in sympathetic ganglia and within the adrenal medulla, with intense TrkB expression restricted to paraganglia, of the normal developing human sympathetic nervous system. In neuroblastoma, ppl40trkA expression correlated positively with favorable tumor stage ($P = 0.0027$) and favorable outcome ($P = 0.026$). No statistically significant correlation of TrkC expression with outcome was evident; however, both TrkA and TrkC expression was most apparent in tumor cells of increased differentiation. **TrkB** expression was primarily localized to cells within the fibrovascular **tumor** stroma. A model of neurotrophin receptor expression and neurotrophin reactivity with differentiation is proposed. The existence and spatial distribution of neurotrophin receptors in neuroblastoma lend supportive evidence that neurotrophic influences may be involved in **tumor** persistence or regression.

... 1995 ,

... however, both TrkA and TrkC expression was most apparent in tumor cells of increased differentiation. **TrkB** expression was primarily localized to cells within the fibrovascular **tumor** stroma. A model of neurotrophin receptor expression and neurotrophin reactivity with differentiation is proposed. The...

...of neurotrophin receptors in neuroblastoma lend supportive evidence that neurotrophic influences may be involved in **tumor** persistence or regression.

...; ME; Ganglioneuroma--pathology--PA; Growth Substances--biosynthesis--BI; Humans; Immunoenzyme Techniques; Infant; Neoplasm Staging; Nerve **Tissue** Proteins--biosynthesis--BI; Neuroblastoma--pathology--PA; Receptor, Ciliary Neurotrophic Factor; Receptor, trkA; Receptor, trkC; Research...

Chemical Name: Growth Substances; Nerve **Tissue** Proteins; Proto-Oncogene Proteins; Receptor, Ciliary Neurotrophic Factor; Receptors, Nerve Growth Factor; Receptor Protein-Tyrosine...

15/3,K,AB/10 (Item 10 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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10229764 PMID: 7972211

Retinoic acid induced differentiation is mediated by trkB receptors.

Lucarelli E; Kaplan D; Matsumoto K; Sickafuse S; Thiele C J

Cell and Molecular Biology Section, NCI/NIH, Bethesda, MD 20892.

Progress in clinical and biological research (UNITED STATES) 1994 ,

385 p185-98, ISSN 0361-7742--Print Journal Code: 7605701

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Retinoic acid induced differentiation is mediated by trkB receptors.

... 1994 ,

...; Derived Neurotrophic Factor; Cell Differentiation--drug effects--DE; Humans; Nerve Growth Factors--pharmacology--PD; Nerve **Tissue** Proteins --pharmacology--PD; Neuroblastoma--drug therapy--DT; Neuroblastoma --pathology--PA; Proto-Oncogene Proteins--metabolism--ME; RNA, Messenger --metabolism--ME; Receptor, trkA; Receptor, **trkB** ; **Tumor** Cells, Cultured ...Enzyme No.: Tyrosine Kinases); EC 2.7.1.112 (Receptor, trkA); EC 2.7.1.112 (Receptor, **trkB**)

Chemical Name: Brain-Derived Neurotrophic Factor; Nerve Growth Factors; Nerve **Tissue** Proteins; Proto-Oncogene Proteins; RNA, Messenger; Receptors, Nerve Growth Factor; Tretinoin; Receptor Protein-Tyrosine Kinases; Receptor, trkA; Receptor, **trkB**

?

Expression of brain-derived neurotrophic factor and tyrosine kinase B receptor proteins in glioneuronal tumors from patients with intractable epilepsy: colocalization with N-methyl-D-aspartic acid receptor.

Aronica E; Leenstra S; Jansen G H; van Veelen C W; Yankaya B; Troost D
Department of (Neuro)Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.
e.aronica@amc.uva.nl

Acta neuropathologica (Germany) Apr 2001 , 101 (4) p383-92, ISSN 0001-6322--Print Journal Code: 0412041

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Recent evidence suggests that brain-derived neurotrophic factor (BDNF) and its tyrosine kinase B (**TrkB**) receptor, in addition to promoting neuronal survival and differentiation, modulates synaptic transmission by increasing N-methyl-D-aspartic acid receptor (NMDAR) activity. Overexpression of BDNF may, then, interfere with normal brain function, causing increased excitability. We have examined the immunohistochemical expression of BDNF, full-length **TrkB** receptor and the NMDAR subunit 1 and subunit 2A/B proteins (NMDAR1 and NMDAR2A/B) in glioneuronal tumors (gangliogliomas, GG, n = 40; dysembryoplastic neuroepithelial tumors , DNT, n = 15), from patients with chronic intractable epilepsy. The great majority of tumors studied were positive for all markers examined, supporting the high level of neurochemical differentiation of these lesions. BDNF and **TrkB** immunoreactivity (ir) was mainly observed in the neuronal component of the tumors . In GG, more than 90% of tumors contained very intense BDNF-ir ganglion cells. Double labeling confirmed the presence of BDNF-ir and **TrkB** -ir in neurons which contained NMDAR1. NMDAR2A/B intensely labeled abnormal neurons in both GG and DNT and co-localized with NMDAR1. The presence of BDNF and its receptor in the neuronal component of GG and DNT may suggest a role for this neurotrophin in the development of these lesions, preventing the death of abnormal neuronal cells. In addition, since these neurons contain both NMDAR1 and NMDAR2A/B subunits, the BDNF- **TrkB** pathway may also contribute through a modulation of glutamatergic transmission to the intrinsic epileptogenicity of glioneuronal tumors .

Expression of brain-derived neurotrophic factor and tyrosine kinase B receptor proteins in glioneuronal tumors from patients with intractable epilepsy: colocalization with N-methyl-D-aspartic acid receptor.

... 2001 ,

Recent evidence suggests that brain-derived neurotrophic factor (BDNF) and its tyrosine kinase B (**TrkB**) receptor, in addition to promoting neuronal survival and differentiation, modulates synaptic transmission by increasing N...

... brain function, causing increased excitability. We have examined the immunohistochemical expression of BDNF, full-length **TrkB** receptor and the NMDAR subunit 1 and subunit 2A/B proteins (NMDAR1 and NMDAR2A/B) in glioneuronal tumors (gangliogliomas, GG, n = 40; dysembryoplastic neuroepithelial tumors , DNT, n = 15), from patients with chronic intractable epilepsy. The great majority of tumors studied were positive for all markers examined, supporting the high level of neurochemical differentiation of these lesions. BDNF and **TrkB** immunoreactivity (ir) was mainly observed in the neuronal component of the tumors . In GG, more than

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... cells. In addition, since these neurons contain both NMDAR1 and NMDAR2A/B subunits, the BDNF- **TrkB** pathway may also contribute through a modulation of glutamatergic transmission to the intrinsic epileptogenicity of glioneuronal **tumors** .

...Descriptors: Epilepsy--metabolism--ME; *Ganglioglioma--metabolism--ME; *Gene Expression Regulation, Neoplastic; *Neoplasm Proteins--analysis--AN; *Nerve **Tissue** Proteins--analysis--AN; *Receptor, **trkB** --analysis--AN; *Receptors, N-Methyl-D-Aspartate--analysis--AN; *Receptors, N-Methyl-D-Aspartate--metabolism...

...; complications--CO; Ganglioglioma--genetics--GE; Humans; Neoplasm Proteins--genetics--GE; Neoplasm Proteins--physiology--PH; Nerve **Tissue** Proteins--genetics--GE; Nerve **Tissue** Proteins--physiology--PH; Neurons --metabolism--ME; Receptor, **trkB** --genetics--GE; Receptor, **trkB** --physiology--PH; Receptors, N-Methyl-D-Aspartate--genetics--GE; Receptors , N-Methyl-D-Aspartate--physiology...

Enzyme No.: EC 2.7.1.112 (Receptor, **trkB**)

...Chemical Name: Derived Neurotrophic Factor; NMDA receptor A1; NR2A NMDA receptor; NR2B NMDA receptor; Neoplasm Proteins; Nerve **Tissue** Proteins; Receptors, N-Methyl-D-Aspartate; Receptor, **trkB**

15/3,K,AB/6 (Item 6 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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12894081 PMID: 11027413

Rational basis for Trk inhibition therapy for prostate cancer .

Weeraratna A T; Arnold J T; George D J; DeMarzo A; Isaacs J T

Johns Hopkins Oncology Center, Johns Hopkins School of Medicine, Baltimore, Maryland, USA.

Prostate (UNITED STATES) Oct 1 2000 , 45 (2) p140-8, ISSN 0270-4137--Print Journal Code: 8101368

Contract/Grant No.: 2P50-CA58236-07; CA; NCI

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

BACKGROUND: Prostatic **cancer** cells are lethal because they acquire the ability to activate survival pathways that do not require androgenic stimulation. As a rational approach to developing effective therapy for these devastating cells, specific signal transduction pathways uniquely required for the survival of these nonandrogen-dependent prostate **cancer** cells must be identified. Previous studies suggested that the neurotrophin/trk signal transduction axis may regulate such unique survival pathways. In the present study, the changes in expression of the neurotrophins (NGF, BDNF, and NT-3) and their cognate receptors (i.e., trk and p75NTR) during the progression of normal prostatic epithelial cells to **malignancy** were documented. Additionally, the consequences of inhibiting these trk signaling pathways on the in vitro survival of prostate **cancer** cells was tested. METHODS: Immunocytochemistry, RT-PCR, and ELISA assays were used to characterize the changes in the neurotrophin ligands (i.e., NGF, BDNF, and NT-3) and their cognate high-affinity (i.e., trk A, B, and

C) and low-affinity neurotrophin (i.e., p75 NTR) receptors in normal vs. **malignant** human prostatic tissues. CEP-751 is an indolocarbazole compound specifically designed to inhibit the initiation of these neurotrophin/trk signal transductions. The consequence of CEP-751 inhibition of trk signaling for in vitro clonogenic survival of a series of human prostatic **cancer** lines was also tested. RESULTS: These studies demonstrated that normal prostatic **tissue** from **patients** without prostate **cancer** contains substantial levels of nerve growth factor (NGF), which is produced in a paracrine manner by stromal cells. These stromal cells lack both trk and p75NTR receptors. In contrast, normal prostatic epithelial cells from **patients** without prostate **cancer** do not secrete detectable levels of neurotrophins, but do express trk A and p75 NTR. While the NGF/trkA/p75 NTR axis is present in the normal prostate, normal prostatic epithelial cells do not depend on this axis for their survival. In contrast, **malignant** prostate epithelial cells directly secrete a series of neurotrophins (i.e., NGF, BDNF, and/or NT-3) and express at least one if not more of the trk receptor proteins (i.e., trk A, B, and/or C), while no longer expressing the p75NTR receptors. In addition, inhibition of autocrine trk signaling via CEP-751 treatment induces the apoptotic death of these **malignant** cells. CONCLUSIONS: Prostate carcinogenesis involves molecular changes leading to the paracrine and/or autocrine production of a series of neurotrophins. This is coupled to the ectopic expression of trk B and trk C, as well as to the continued expression of trk A, and the loss of expression of p75NTR receptors. These changes result in the acquisition by **malignant** prostate cells of a unique requirement for trk signaling pathways for survival. Based on these findings, trk inhibition is a novel, rational approach for prostate **cancer** therapy. Copyright 2000 Wiley-Liss, Inc.

Rational basis for Trk inhibition therapy for prostate cancer .

... 2000 ,

BACKGROUND: Prostatic **cancer** cells are lethal because they acquire the ability to activate survival pathways that do not...

... cells, specific signal transduction pathways uniquely required for the survival of these nonandrogen-dependent prostate **cancer** cells must be identified. Previous studies suggested that the neurotrophin/trk signal transduction axis may...

... receptors (i.e., trk and p75NTR) during the progression of normal prostatic epithelial cells to **malignancy** were documented. Additionally, the consequences of inhibiting these trk signaling pathways on the in vitro survival of prostate **cancer** cells was tested. METHODS: Immunocytochemistry, RT-PCR, and ELISA assays were used to characterize the...

... B, and C) and low-affinity neurotrophin (i.e., p75 NTR) receptors in normal vs. **malignant** human prostatic tissues. CEP-751 is an indolocarbazole compound specifically designed to inhibit the initiation...

...inhibition of trk signaling for in vitro clonogenic survival of a series of human prostatic **cancer** lines was also tested. RESULTS: Th

? ds

Set	Items	Description
S1	6358	TRKB
S2	2472	NT4 OR (NT(W)4)
S3	5519	S1 NOT S2
S4	3432215	CANCER? OR TUMOR? OR MALIGNAN?
S5	575	S3 AND S4
S6	479099	NECROSIS
S7	501	S5 NOT S6
S8	378	RD (unique items)
S9	314	S8 AND PY<=2003
S10	1045564	CELL(W)LINE??
S11	219	S9 NOT S10
S12	8327234	TISSUE OR PATIENT??
S13	84	S11 AND S12
S14	636563	MRNA
S15	72	S13 NOT S14

? ds

Set	Items	Description
S1	6358	TRKB
S2	2472	NT4 OR (NT(W)4)
S3	5519	S1 NOT S2
S4	3432215	CANCER? OR TUMOR? OR MALIGNAN?
S5	575	S3 AND S4
S6	479099	NECROSIS
S7	501	S5 NOT S6
S8	378	RD (unique items)
S9	314	S8 AND PY<=2003
S10	1045564	CELL(W)LINE??
S11	219	S9 NOT S10
S12	8327234	TISSUE OR PATIENT??
S13	84	S11 AND S12
S14	636563	MRNA
S15	72	S13 NOT S14

? s review

S16 1207442 REVIEW

? s s15 and s16

72 S15

1207442 S16

S17 5 S15 AND S16

? t s17/3,k,ab/1-5

17/3,K,AB/1 (Item 1 from file: 55)

DIALOG(R)File 55:Biosis Previews(R)

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0014452668 BIOSIS NO.: 200300421387

Neuroblastoma: A developmental perspective.

AUTHOR: McConville Carmel M (Reprint); Forsyth Joan

AUTHOR ADDRESS: Department of Paediatrics and Child Health, University of Birmingham, Vincent Drive, Birmingham, B15 2TT, UK**UK

AUTHOR E-MAIL ADDRESS: c.mcconville@bham.ac.uk

JOURNAL: Cancer Letters 197 (1-2): p3-9 July 18, 2003 2003

MEDIUM: print

ISSN: 0304-3835 (ISSN print)

DOCUMENT TYPE: Article; Literature Review

RECORD TYPE: Abstract

LANGUAGE: English

ABSTRACT: Genetic investigation of neuroblastoma has provided few clues to account for the variability in clinical phenotype which is such a characteristic feature of this tumour. Indeed, efforts to identify the primary genetic event(s) responsible for tumour development have been overwhelmed by the number and range of different genetic abnormalities observed, particularly in the more aggressive neuroblastoma subtypes. Since neuroblastoma is a consequence of aberrant development of the sympathetic nervous system (SNS), investigation of the genetic components known to be involved in the control of SNS development, may provide the key to understanding tumour behaviour. The neurotrophins and the glial family ligands both play very significant roles in different stages of SNS development and merit more detailed investigation as to how they might influence neuroblastoma **tumorigenesis** .

2003

...ABSTRACT: of SNS development and merit more detailed investigation as to how they might influence neuroblastoma **tumorigenesis** .

DESCRIPTORS:

...MAJOR CONCEPTS: **Tumor** Biology
...ORGANISMS: PARTS ETC: extra-embryonic **tissue** ;
CHEMICALS & BIOCHEMICALS: ... **TrkB** ;
MISCELLANEOUS TERMS: ... **tumorigenesis** ; ...

...Literature **Review**

17/3,K,AB/2 (Item 1 from file: 34)

DIALOG(R) File 34:SciSearch(R) Cited Ref Sci

(c) 2006 The Thomson Corp. All rts. reserv..

02100509 Genuine Article#: KA963 Number of References: 120

Title: **BIOCHEMICAL-STUDY OF CYST FLUID IN HUMAN BREAST CYSTIC-DISEASE - A REVIEW** (Abstract Available)

Author(s): ENRIORI CL; NOVELLI JE; CREMONA MD; HIRSIG RJP; ENRIORI PJ

Corporate Source: LAB ANAL CLIN,CORDOBA 2077/RA-1120 BUENOS

AIRES//ARGENTINA//; HOSP FRANCES RIOSA,SERV GINECOL &

MASTOL/BUENOSAIRE//ARGENTINA//; HOSP COSME ARGERICH,SERV CIRUGIA/BUENOS AIRES//ARGENTINA/

Journal: BREAST CANCER RESEARCH AND TREATMENT, 1992 , V24, N1, P1-9

ISSN: 0167-6806

Language: ENGLISH Document Type: REVIEW

Abstract: Gross cystic disease of the breast may sometimes indicate an increased risk of breast **cancer** . Biochemical analysis of the cyst fluid could suggest which cysts are associated with breast **cancer** risk, as well as providing insights into the pathophysiology of this condition. The Na⁺/K⁺ ratio appears to be associated with the histological classification of the cyst. Sulfoconjugated estrogens and androgens, especially DHEA-S, are often found at high levels. A number of gross cystic disease fluid proteins (GCDFPs) have been described, and several polypeptide growth factors including EGF and IGF-I are frequently found. It is hoped that biochemical analysis of these components of breast cyst fluids will shed further light on the role of gross cysts in relation to breast **cancer** .

Title: BIOCHEMICAL-STUDY OF CYST FLUID IN HUMAN BREAST CYSTIC-DISEASE - A REVIEW
, 1992

Abstract: Gross cystic disease of the breast may sometimes indicate an increased risk of breast **cancer** . Biochemical analysis of the cyst fluid could suggest which cysts are associated with breast **cancer** risk, as well as providing insights into the pathophysiology of this condition. The Na+/K...

...fluids will shed further light on the role of gross cysts in relation to breast **cancer** .

...Identifiers--EPIDERMAL-GROWTH-FACTOR; TERM **TISSUE** -CULTURE; FACTOR-I; HUMAN-MILK; FIBROCYSTIC DISEASE; FACTOR RECEPTORS; SOMATOMEDIN-C; **CANCER** -CELLS; HUMAN-PLASMA; DEHYDROEPIANDROSTERONE-SULFATE

...Research Fronts: PEPTIDE SECRETION)

90-3735 002 (EPIDERMAL GROWTH-FACTOR; EXPRESSION OF THE TYROSINE KINASE RECEPTOR GENE **TRKB** ; MURINE KIDNEY MEMBRANES)

90-7814 002 (EPIDERMAL GROWTH-FACTOR RECEPTOR; MONOCLONAL EGFR1 ANTIBODY IN PRIMARY BREAST- **CANCER** **PATIENTS** ; C-ERBB2 EXPRESSION)

90-2019 001 (NODE-NEGATIVE BREAST- **CANCER** ; LONG-TERM ADJUVANT TAMOXIFEN THERAPY; PROGNOSTIC FACTORS; FLOW CYTOMETRIC DNA ANALYSIS)

90-2081 001 (INSULIN...

17/3,K,AB/3 (Item 2 from file: 34)

DIALOG(R) File 34:SciSearch(R) Cited Ref Sci

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01717607 Genuine Article#: HV394 Number of References: 74

Title: ROLE OF EPIDERMAL GROWTH-FACTOR IN OBSTETRICS AND GYNECOLOGY (Abstract Available)

Author(s): MIYAZAWA K

Corporate Source: UNIFORMED SERV UNIV HLTH SCI,DEPT OBSTET & GYNECOL,4301 JONES BRIDGE RD/BETHESDA//MD/20814

Journal: OBSTETRICS AND GYNECOLOGY, **1992** , V79, N6 (JUN), P1032-1040

Language: ENGLISH Document Type: REVIEW

Abstract: During the past 30 years, a number of growth factors have been isolated and characterized. These include insulin and insulin-like growth factors, the fibroblast growth factors, the hematopoietic colony-stimulating growth factors, and the epidermal growth factor (EGF) group. Epidermal growth factor, which stimulates the growth of a variety of tissues, has been extensively studied since its discovery in the early 1960s. This **review** presents an historic perspective, basic scientific aspects, laboratory methods, and the clinical application of EGF in the specialty of obstetrics

29/3,K,AB/1 (Item 1 from file: 155)
DIALOG(R) File 155:MEDLINE(R)
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12522750 PMID: 10468769

Expression of p75(LNGFR) and Trk neurotrophin receptors in normal and neoplastic human prostate.

Guate J L; Fernandez N; Lanzas J M; Escaf S; Vega J A
Servicios de Urologia Hospital San Agustin, Aviles; Departamentos de Cirugia y Especialidades Medico-Quirurgicas. Madrid, Spain.

BJU international (ENGLAND) Sep 1999 , 84 (4) p495-502, ISSN 1464-4096--Print Journal Code: 100886721

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

OBJECTIVE: To analyse the occurrence and cell distribution of p75(LNGFR) and Trk neurotrophin receptors in normal prostate, benign prostatic hypertrophy (BPH) and prostate carcinoma, and to determine the effect of androgen suppression on the expression of these proteins in prostate **cancer** samples. MATERIALS AND METHODS: The study comprised formalin-fixed and paraffin-embedded material, obtained during surgery and from cadavers during removal of organs for transplantation. Light microscopy immunohistochemistry was carried out using polyclonal antibodies against Trks, and a monoclonal antibody against p75(LNGFR). General markers for epithelial and endocrine cells were assessed in parallel. RESULTS: TrkA immunoreactivity (IR) was restricted to the basal epithelial cells in some acini (37%). This pattern remained unchanged or IR extended to the whole acini in BPH, and varied widely in prostate **cancer**. In normal **tissue** and BPH, TrkC IR was **detected** exclusively in the stroma. Nevertheless, it progressively increased in the epithelial cells of well-differentiated to moderately differentiated prostate carcinoma, whereas in stromal cells there were no substantial changes. **TrkB** IR was absent in all the samples. There was weak p75(LNGFR) IR in normal epithelial cells, which increased in prostate **cancer** and to a lesser extent in BPH. Androgen suppression was ineffective in reversing TrkA modifications, whereas it caused a decrease in the expression of TrkC and p75(LNGFR). CONCLUSION: The abnormal growth of prostatic epithelium is accompanied by increased TrkA expression and the induction of TrkC expression in epithelial cells. These results suggest that neurotrophins could be involved in the abnormal growth of the human prostate, acting through specific Trk signal-transducing receptors whose expression is regulated by androgens.

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... to determine the effect of androgen suppression on the expression of these proteins in prostate **cancer** samples. MATERIALS AND METHODS: The study comprised formalin-fixed and paraffin-embedded material, obtained during...

...unchanged or IR extended to the whole acini in BPH, and varied widely in prostate **cancer**. In normal **tissue** and BPH, TrkC IR was **detected** exclusively in the stroma. Nevertheless, it progressively increased in the epithelial cells of well-differentiated to moderately differentiated prostate carcinoma, whereas in stromal cells there were no substantial changes. **TrkB** IR was absent in all the samples. There was weak p75(LNGFR) IR in normal epithelial cells, which increased in prostate **cancer** and to a lesser extent in BPH. Androgen suppression was ineffective in reversing

TrkA modifications...

29/3,K,AB/2 (Item 1 from file: 55)

DIALOG(R)File 55:Biosis Previews(R)

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**EPILEPSY - INDUCED CHANGES IN POSTSYNAPTIC TRKB , NMDA RECEPTOR AND
PSD95/SAP90 PROTEINS.**

AUTHOR: Marengo J (Reprint); Wyneken U; Sandoval R; Gundelfinger E; Orrego
F

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? ds

Set	Items	Description
S1	6358	TRKB
S2	2472	NT4 OR (NT(W)4)
S3	5519	S1 NOT S2
S4	3432215	CANCER? OR TUMOR? OR MALIGNAN?
S5	575	S3 AND S4
S6	479099	NECROSIS
S7	501	S5 NOT S6
S8	378	RD (unique items)
S9	314	S8 AND PY<=2003
S10	1045564	CELL(W)LINE??
S11	219	S9 NOT S10
S12	8327234	TISSUE OR PATIENT??
S13	84	S11 AND S12
S14	636563	MRNA
S15	72	S13 NOT S14
S16	1207442	REVIEW
S17	5	S15 AND S16
S18	134	TRKB (5N) LABEL?
S19	0	S15 AND S18
S20	195	TRKB (5N) (PROBE OR LIGAND)
S21	0	S15 AND S20
S22	2472	NT4 OR (NT(W)4)
S23	0	S15 AND S22
S24	192137	SUPPRESSOR
S25	68	S15 NOT S24

? s treat?

Processing

S26 5967492 TREAT?

? s s25 not s26

68 S25

5967492 S26

S27 59 S25 NOT S26

? s detect? or diagnos?

Processing

3486928 DETECT?

3848381 DIAGNOS?

S28 6807923 DETECT? OR DIAGNOS?

? s s27 and s28

59 S27

6807923 S28

S29 23 S27 AND S28

? t s29/3,k,ab/1-23

29/3,K,AB/1 (Item 1 from file: 155)

DIALOG(R) File 155:MEDLINE(R)

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12522750 PMID: 10468769

Expression of p75(LNGFR) and Trk ne